Coronary effects of endothelins

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Coronary Effects of Endothelins

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SUMMARY

The endothelins, recently discovered potent vasoconstrictor peptides, may be implicated in detrimental effects in cardiac ischaemia. Studies in the isolated rat heart show an increase in both their synthesis and binding following simulated ischaemia and reperfusion. An increase in binding density does not necessarily correlate with an increase in numbers of functional receptors. Hence studies were performed to confirm any selective potentiation of endothelin effect, using a similar protocol of ischaemia and reperfusion to that producing an increase in binding.

An apparent increase in the coronary constrictor effect of endothelins was shown to follow ischaemia and reperfusion, accompanied by loss of their initial vasodilator action. This increase was, however, inconclusive as rising basal perfusion pressure in ischaemic / reperfused hearts made interpretation difficult. Desensitisation of the coronary dilator component of endothelin responses (using an ET_B receptor agonist, sarafotoxin 6c) revealed that loss of opposing vasodilatation alone is sufficient to enhance the vasoconstrictor action of these peptides.

The relative potencies of the endothelin family of peptides and use of a selective ET_B receptor agonist and a selective ET_A receptor antagonist showed that coronary dilatation was consistent with action via ET_B receptors, whereas vasoconstriction was consistent with action through an ET_A receptor.

An increase in intracellular calcium is an important signal transduction mechanism for endothelins, although reports of the dynamics of the increase include some inconsistencies which may depend on the model used. The effect of endothelin-1 on calcium signalling in cultured porcine coronary smooth muscle cells was investigated by fluorimetric assay using fura-2. Responses measured in cells in confluent monolayers, were compared with responses in single cells and in dispersed cell suspensions.

Cells from different hearts displayed variability in calcium responses in monolayers, but these usually included a peak and a plateau phase of response as previously reported. Single cell responses were more inconsistent, with variability in the form of responses in

cells from the same heart. These included an initial peak followed by variable oscillations. Responses in suspensions of large cell numbers were less variable, including a peak and a plateau phase. However, different patterns of inhibition by BQ-123 were seen in monolayers and in suspensions.

In conclusion, selection of the type of cell preparation is important for comparison of effect and a larger experimental number is required for investigations in primary cell cultures.

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INTRODUCTION

1.1 Coronary heart disease

Epidemiological studies demonstrate that death from coronary heart disease is a major problem in the Western world, with England and Wales being among the worst affected countries, and Scotland and Northern Ireland having the highest death rates (Thom, 1989). This disease is usually a result of narrowing of the coronary arteries, and may be manifested initially by angina pectoris (chest pain on exertion), where an increase in demand over myocardial blood supply leads to ischaemia of the affected area of myocardium. Myocardial infarction (death of cardiac tissue) is the result of longer lasting coronary obstruction, and can be the result of a coronary thrombosis, i.e. total interruption of supply to the area fed by the coronary artery concerned. However, the first indication of disease may be the event of sudden cardiac death, where complete loss of cardiac output may be the result of an arrhythmia (Lorimer & Hills, 1985). The incidence and severity of coronary heart disease have ensured considerable ongoing research activity.

1.1.1 Anatomy

The coronary vessels leave the aortic trunk as two main arteries from the left and right of the aorta, immediately above the aortic valve. These follow the epicardial surface and as they branch, smaller vessels enter the myocardium and endocardium and form the supply to the whole of the left and right atria and ventricles of the heart. The size of these arteries and arterioles gradually decreases from the larger conductance, to the small resistance vessels, the size of which may be regulated by neural and humoral factors. The major coronary arteries may be seen in figure I-1.

THE CORONARY CIRCULATION

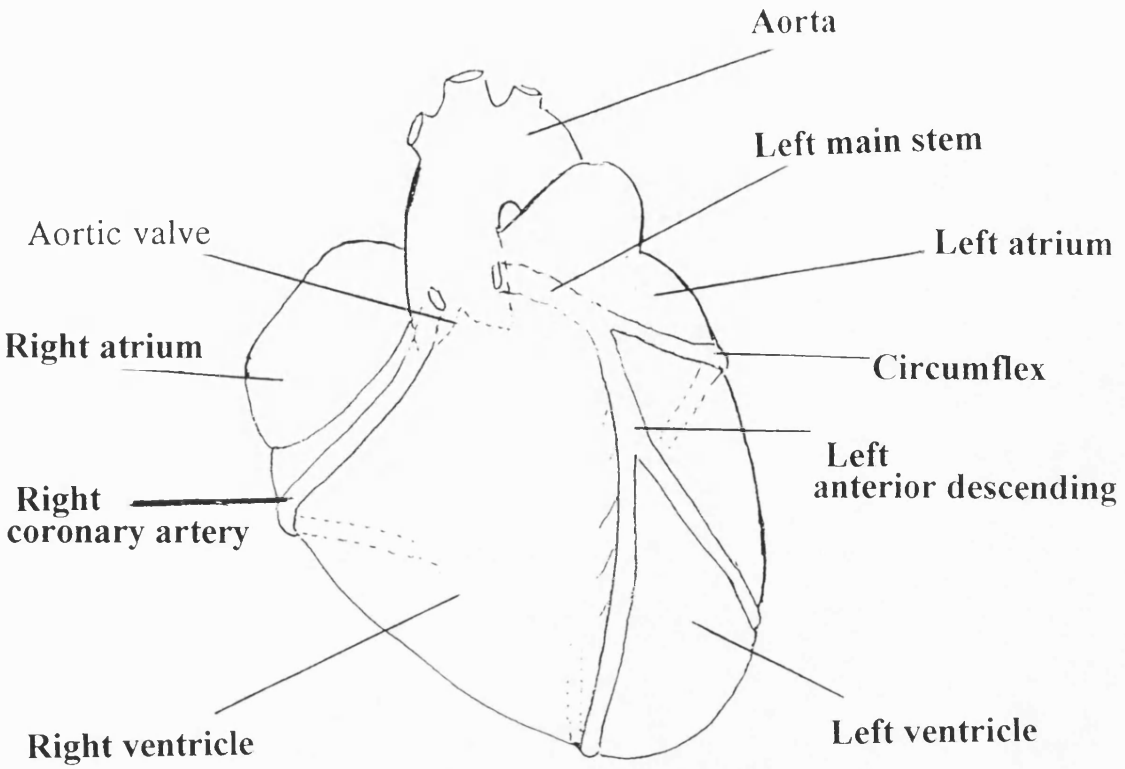


Figure I-1

The human coronary circulation showing the anatomical position of the major branches of the left and right coronary arteries.

1.1.2 Ischaemic changes

The oxygenated blood supplied to the myocardium by the coronary circulation allows energy for contraction of the muscle to be provided by oxidative phosphorylation, where adenosine triphosphate (ATP) production is aerobic. Coronary insufficiency leads to anaerobic metabolism and the build up of acidic metabolites. The symptom most commonly exhibited as a result of this ischaemia is angina pectoris (chest pain).

Ischaemic damage to the cardiac muscle cells leads also to extracellular accumulation of potassium ions and thus local cell membrane depolarisation. Disruption of the normal membrane potential, which in turn may reduce the ability of the cells to conduct action potentials, may also result in the abnormal initiation of action potentials. The end product is the loss of ordered conduction of normal cardiac rhythm which is necessary for coordinated myocardial contraction, and this can result in a loss of cardiac output which is life-threatening.

Coronary narrowing is usually caused by atheromatous plaques which may also disrupt the smooth endothelial layer, leaving a surface upon which platelets may aggregate, and leading to thrombus formation obstructing the artery concerned. This results in myocardial infarction if untreated, reperfusion of the affected area being an immediate aim of therapy to prevent cellular necrosis. Narrowing may also be caused by, or exacerbated by coronary spasm, again leading to the possibility of coagulation due to the disruption of laminar flow of blood. This variant angina may occur transiently with ischaemia usually followed by reperfusion of the myocardial tissue on recovery.

1.1.3 Current treatments - medical and surgical

The current treatments for coronary artery narrowing are, in the first instance, reduction of cardiac oxygen demand using vasodilator agents and ß-adrenergic antagonists, both of which reduce the oxygen demands of the heart. This may be accompanied by use of anticoagulant therapy to prevent thrombus formation. Where medical treatment fails to reduce angina pectoris or the risk of thrombosis is seen to be great, attempts are made to remove the stenoses. This can be achieved using the percutaneous transluminal coronary

angioplasty technique, which employs inflation of a balloon to compress the atheromatous plaque into the vessel wall. This procedure may be accompanied by the use of a stent to maintain the patency of the vessel. The surgical alternative to this procedure is coronary artery bypass, usually using a graft of saphenous vein or internal mammary artery to restore blood supply to the ischaemic myocardium. The more invasive open heart surgery has been shown to be more effective in avoiding recurrence (King *et al.*, 1994), although the procedure carries greater immediate risk than angioplasty.

Treatment is available for recently occurring thrombosis, allowing salvage of myocardium at risk of necrosis. This increasingly involves the use of thrombolytic agents which, if used in the early stages of myocardial infarction, can restore perfusion and prevent or limit the progress of cellular necrosis. However, this procedure has been shown to carry several risks, including that of reperfusion arrhythmias, and inadequate reperfusion of the area at risk of infarction, the "no reflow" phenomenon (Kloner *et al.*, 1974). The reperfused area may not recover its function, an occurrence known as myocardial stunning (e.g. Triana & Bolli, 1991).

1.1.4 Reperfusion

Ischaemia itself causes severe cellular damage and necrosis but the restoration of blood flow is, paradoxically, an initiator of more damaging processes. Much of the damage to cardiomyocytes leading to contractile dysfunction, is believed to be related to the intracellular accumulation of calcium ions. The increased uptake of these ions correlates with depressed recovery of contractility after reperfusion of the ischaemic heart (Tani, 1990). This calcium overload is related to the development of ischaemic contracture in experimental hearts.

Coronary damage is also exacerbated by reperfusion. Lack of reflow is a result of changes in coronary vessels which include inappropriate vasospasm, loss of vasodilator function, and endothelial oedema and functional loss (Katz, 1992; Sobey & Woodman, 1993). The latter may involve accumulation, on reperfusion, of inflammatory cells such as neutrophils which can physically block vessels, and which also release factors (including superoxide anion)

which exacerbate endothelial cell damage. This may be accompanied by platelet aggregation in the damaged vessels, a process exacerbated by loss of the inhibitory presence of factors such as nitric oxide (NO) and prostacyclin (PGI_2), production of which may be compromised in post-ischaemic endothelium (Sobey & Woodman, 1993). The degree of damage and functional loss is related to the duration of the ischaemic episode in experimental models (Kloner *et al.*, 1974; Nevalainen *et al.*, 1986; Viehman *et al.*, 1991). Among the local mediators which have been shown to contribute to coronary arterial spasm are a family of recently-discovered peptides, the endothelins (Kurihara *et al.*, 1989).

1.2 Endothelins

1.2.1 Discovery of a novel vasoconstrictor peptide

It has become increasingly clear that the endothelium, once considered as simply a smooth physical barrier, plays an important role in the production of factors involved in the regulation of vascular tone. For example, the cells of the endothelial layer have been shown to produce vasodilator eicosanoids (PGI_2, PGE_2) and ATP (reviewed in Dale & Foreman, 1989) and endothelium-derived relaxing factor (EDRF, Furchgott & Zawadski, 1980) which act on the underlying vascular smooth muscle cells to produce changes locally in the diameter of the vessel. The discovery of EDRF was followed by the finding that endothelium produces a peptide constricting factor (Hickey *et al.*, 1985), and this novel factor was further identified and the gene cloned and sequenced by Yanagisawa *et al.*, (1988). The peptide (relative molecular mass 2492) was isolated from supernatants of porcine aortic endothelial cells and purified, before being identified as a 21 amino acid structure. This "endothelin" was noted to be the most potent naturally occurring vasoconstrictor yet discovered, having an EC_{50} of 0.4nM in various arterial preparations (Yanagisawa *et al.*, 1988) and producing a long-lasting (>20minutes) vasoconstriction and pressor effect. A transient vasodilator effect was also seen prior to vasoconstriction in some preparations in these initial studies. The peptide discovered by Yanagisawa's group was later designated "endothelin-1", the only endothelin to be produced by endothelium. A preproendothelin of approximately 200 amino acid residues and a 38 amino acid pro-endothelin, "big endothelin", were also determined and a synthetic pathway involving an endothelin converting enzyme (ECE) was proposed (Yanagisawa *et al.*, 1988; see figure I-2). The production of endothelin-1 was found to be by *de novo* synthesis, its release being dependent on the microtubular system (Kitazumi *et al.*, 1991).

Subsequent research demonstrated the existence of other endothelin species, designated endothelin-2 and endothelin-3 (Inoue *et al.*, 1989), all products of separate genes. The three peptides share the bicyclic structure produced by four cysteine residues (at positions 1,3,11 and 15) which form disulphide bonds 1-15 and 3-11. These bonds suggest

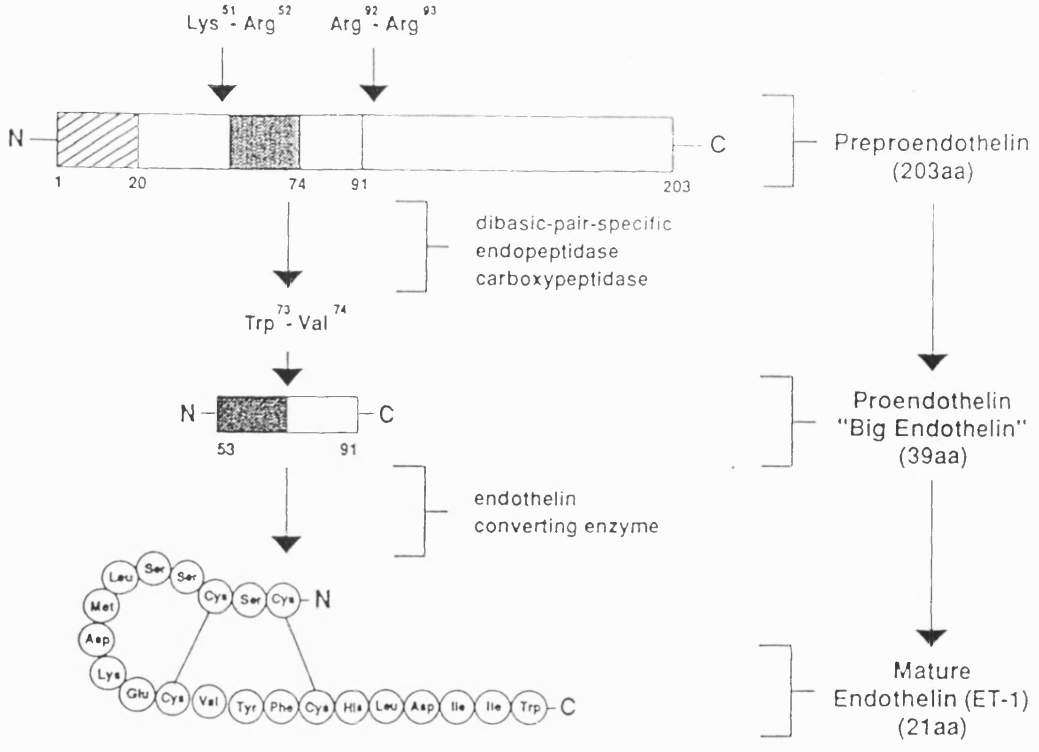


Figure I-2

The synthetic pathway of endothelin-1, demonstrating the post-translational proteolytic processing of the peptide. (From Highsmith *et al.*, 1992).

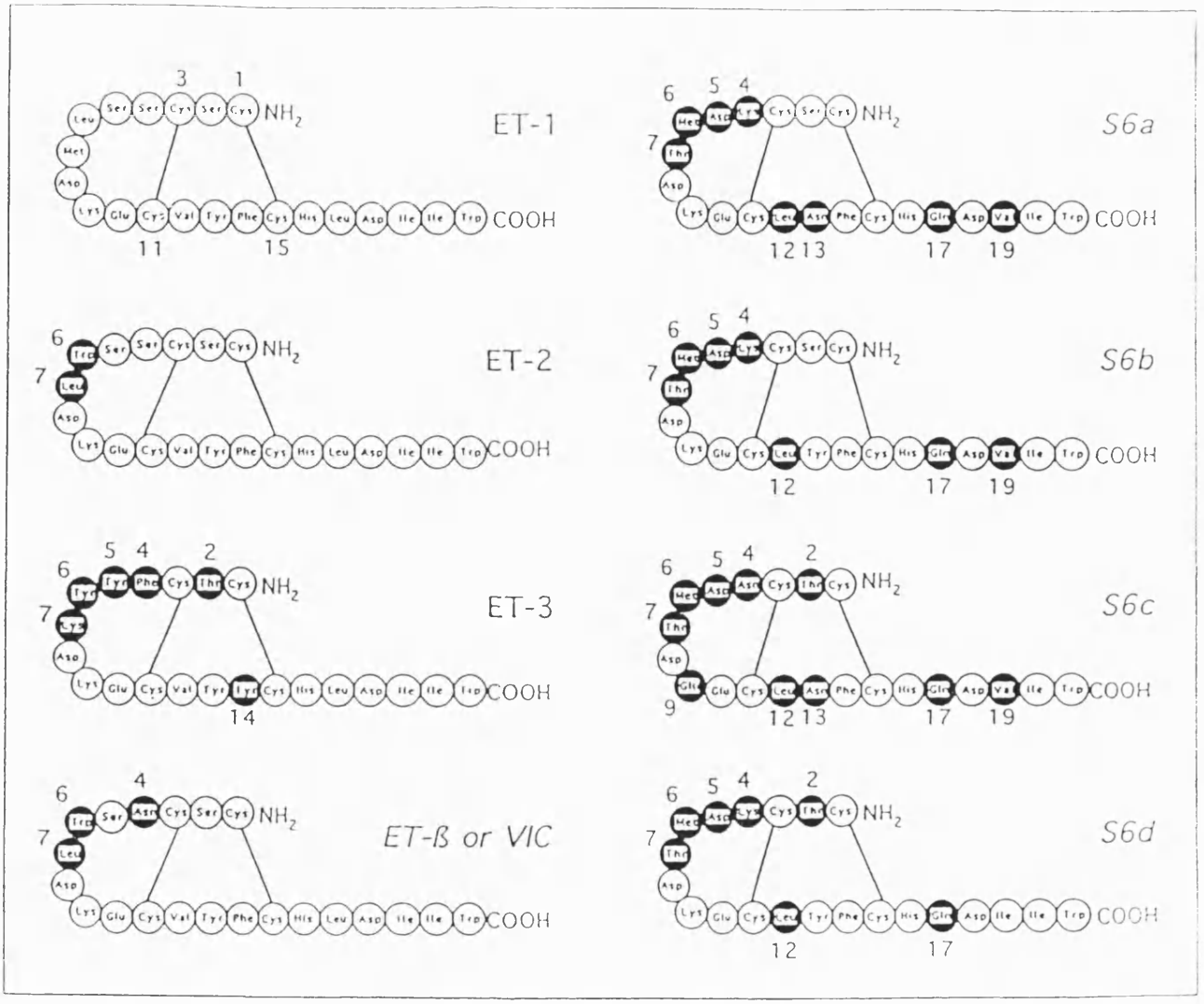


Figure I-3
Structures of endothelins and the related snake venom-derived sarafotoxins. Homologous amino acid residues are shown as open circles, while those differing from endothelin-1 are shown as dark circles. (Figure shows structures described by Yanagisawa *et al.*, 1988 (ET-1), Inoue *et al.*, 1989 (ET-2, ET-3), Saida *et al.*, 1989 (VIC) and Kloog & Sokolovsky, 1989 (sarafotoxins)).

that the peptides form a helical structure (Sokolovsky, 1992). The main differences in their amino acid sequences occur between amino acid residues in the loop between the inner disulphide bonds (see figure I-3). The endothelin peptides bear close sequence homology to murine vasoactive intestinal constricting substance (VIC, Nayler, 1990) and to the sarafotoxins, a family of snake venoms isolated from *Actractaspis engaddensis* (Kloog & Sokolovsky, 1989).

The cleavage site for the formation of the three endothelin isopeptides from their 38-39 amino acid precursor "big endothelins", is unusual, occurring between valine and tryptophan residues. Enzyme activity performing this function has been found in endothelial cells as well as in the membrane fraction of vascular smooth muscle cells (Hisaki *et al.*, 1993; Tsukahara *et al.*, 1993), suggesting a local function for the active peptide. Cleavage from big endothelin is important for full vasoconstrictor function (Kimura *et al.*, 1989), and is sensitive to metalloprotease inhibitors such as phosphoramidon.

1.2.2 Endothelin synthesis and release

The mRNA for all three endothelin isopeptides appears to be widespread, found in all mammals tested (Inoue *et al.*, 1989) and in most organs in varying concentrations, although endothelin-2 is possibly less ubiquitous than the other isopeptides (Firth and Ratcliffe, 1992). A variant of endothelin-2, vasoactive intestinal peptide (see figure I-3) is also found in mice. However, all three endothelins have been reported as present in human heart (Plumpton *et al.*, 1993), and endothelin species have been detected in human coronary arteries and veins (Opgaard *et al.*, 1994).

Endothelin-1 is not stored in cells and is constitutively produced by endothelium at a basal level (Dickinson *et al.*, 1991). The expression of its mRNA in cultured endothelial cells may be induced following stimulation by a variety of factors, including angiotensin II (Kohno *et al.*, 1992), the calcium ionophore A23187 and thrombin, e.g. from aggregating platelets (Kurihara *et al.*, 1989). The thrombin- and A23187-stimulated production of endothelin-1 from endothelium of intact porcine aortae is inhibited by NO (Boulanger & Lüscher, 1990) and by atrial and brain natriuretic peptides (Kohno *et al.*, 1992), via a cyclic

guanosine 5'-monophosphate (cGMP)-sensitive mechanism. This may suggest that the local regulation of vessel size is influenced by the balance of these mediators. Release of endothelins has been shown to occur mainly basolaterally, again supporting a role for the peptide as a local mediator (Wagner *et al.*, 1992).

Rat and rabbit aortic vascular smooth muscle cells in culture have also been shown to release endothelin-1, but at lower concentrations than endothelial cells (in the range 0.1-1 compared with 10-80 fmol /10^5 cells/24 hours; Kanse *et al.*, 1991). Suppression of this basal endothelin-1 release from smooth muscle (but not endothelial cells) has been shown to be cyclic AMP-sensitive.

It has also been demonstrated that low density lipoproteins in human and porcine tissues (Boulanger *et al.*, 1992) and hypoxia in rat lung (Shirakami *et al.*, 1991), can trigger the production of endothelins. These findings suggest that these peptides could be induced, and therefore may have a role to play in arterial pathology.

1.2.3 Degradation

Circulating endothelins are cleared rapidly from the blood. This may occur at or near the site of production, as the vasculature itself may possess a neutral endopeptidase which is phosphoramidon sensitive and actively metabolises endothelins (Dickinson *et al.*, 1991) . Extracts of lung have also been shown to degrade the peptide efficiently. The renal contribution to degradation may be of greatest importance under normal conditions (Rubanyi & Polokoff, 1994); a carboxypeptidase from soluble renal extracts has been shown to cleave the carboxyl terminal of the endothelin-1 molecule thus rendering it inactive (Jeng & Deng, 1993). In other organs enzymes could contribute to catabolism possibly with variations in optimal pH; for example, the degradation of the peptide by myocardial extract was found to be more efficient at low pH (6.2), suggesting that removal mechanisms may be enhanced in ischaemic conditions (Garjani *et al.*, 1994).

1.3 Endothelin Receptors

The vasodilator and vasoconstrictor components of the vascular response to endothelins can be separated according to the order of potency of the isopeptides and sarafotoxins which provoke them. The vasoconstrictor component has been shown to respond with sensitivity as follows: ET-1>ET-2>ET-3>Sx6c, whereas the vasodilator response is equally sensitive to all three endothelins and sarafotoxin 6c. These results suggest activation of two distinct receptors and this has been confirmed by further characterisation of two receptors, one from bovine lung (designated ET_A, Arai *et al.*, 1990) and one from rat lung preparations (designated ET_B, Sakurai *et al.*, 1990), which show different pharmacological profiles. The ET_A receptor corresponds to the potency response profile for vasoconstriction (ET-1=Sx6b >ET-2 >> ET-3), whereas the ET_B receptor mediates vasodilatation with equal sensitivity to the endothelins and sarafotoxins (ET-1 = ET-2 = ET-3 = Sx6b).

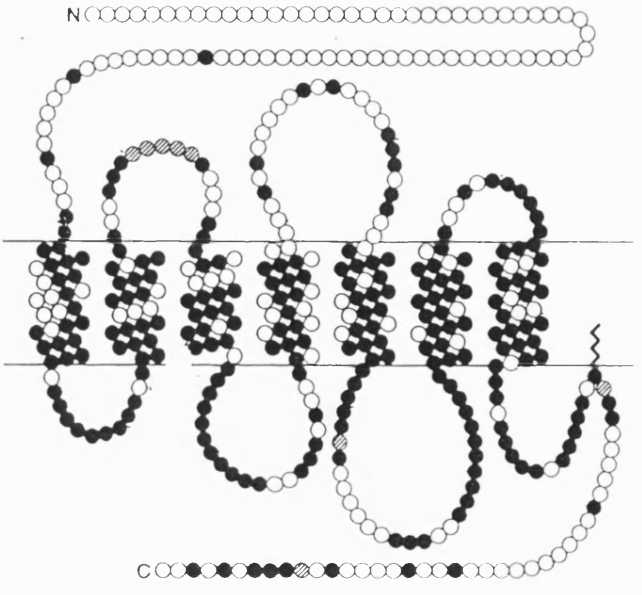
The receptors cloned were both of a structure comprising seven hydrophobic domains, likely to be membrane spanning regions, and having a probable extracellular N-terminus and an intracellular C-terminus (figure I-4). The seven membrane-spanning domains are a feature characteristic of the rhodopsin-like receptor family which are linked to GTP-binding proteins (G-proteins - see section 1.6.1).

The endothelins bind to the ET_A and ET_B receptors with differing affinities which reflect the relative potencies described above with affinity constants as follows:

(From Sakamoto *et al.*, 1993, and Williams *et al.*, 1993).

Receptor	**Affinity: K_i (nM)**		
	Endothelin-1	Endothelin-3	Sarafotoxin 6c
ET_A	3.5	1000	2800
ET_B	0.95	2.0	0.29

Extracellular domain



Intracellular domain

Figure I-4

From Sakamoto *et al.*, 1993. Predicted structure of the endothelin receptors. The wavy line denotes a likely palmitoylation site, closed circles those amino acid residues homologous between ET_A and ET_B receptors.

The dissociation of the endothelins from their receptors has been shown to be extremely slow in vascular tissues, in common with other preparations (Hemsén *et al.*, 1991) and this is complicated by the internalisation of ligand-receptor complexes (Bax & Saxena, 1994).

The binding of endothelins to either receptor is dependent on the presence of the C-terminal Trp^{21} residue, and studies using chimaeric receptors have demonstrated that the tertiary structure of the molecule is important for the binding of peptides to the ET_A and ET_B receptors: the highly conserved linear C-terminal "tail" has been shown to bind to either ET_A or ET_B receptors, whereas both the helically coiled "head" and the linear tail are necessary for the binding of the peptides to the ET_A receptor (Sakamoto *et al.*, 1993). The integrity of the disulphide bonds, and hence the helical structure is also important for the vasoconstrictor activity of the peptides (Cody *et al.*, 1991). Much of this information has been deduced using analogues of the peptide, which have been shown to be selective for either ET_A or ET_B receptors. These include amino-terminally truncated linear endothelin analogues which bind selectively to the ET_B receptor such as BQ-3020 and IRL 1620, where they act as agonists (see Haynes *et al.*, 1993).

Several tools have been developed for the study of the receptor subtypes. ET_A receptor antagonists have been produced including the cyclic pentapeptide BQ-123 (cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-), which was developed from products of the culture of *Streptomyces misakiensis* (Ihara *et al.*, 1992). This has a pA_2 value measured at 7.4 against endothelin-1-induced vasoconstriction in porcine coronary artery, and has been one of the most widely used ET_A-selective antagonists, although non-peptide antagonists have now been produced. The development of ET_B-selective antagonists has been more problematic and has taken longer to achieve. ET_B-selective agonists such as IRL 1620 (Takai *et al.*, 1992) mentioned above, and sarafotoxin 6c (Williams *et al.*, 1991), have instead been used for the study of receptor subtypes, as well as non-peptide, selective and non-selective endothelin antagonists such as PD 142893, PD 145065, FR 139317 (ET_A), Ro 46-2005, SB 209670, and BMS 1822874, and bosentan (e.g. Buchan *et al.*, 1994; Gardiner *et al.*, 1994). More recently a peptide, BQ-788, (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) has been described as a novel selective

antagonist at the ET_B receptor with a pA_2 of 8.4 (measured against pulmonary artery vasoconstriction to BQ-3020; Ishikawa *et al.*, 1994).

There is evidence of at least one further endothelin receptor, which is selective for endothelin-3. Stimulation of this receptor results in the inhibition of prolactin release from cultured anterior pituitary cells, and endothelin-3 was shown to be effective where endothelin-1 was not (Samson *et al.*, 1990). A separate study has identified an endothelin-3-selective receptor in cultured bovine endothelial cells (Emori *et al.*, 1990). An endothelin-3-specific receptor has since been cloned from amphibian dermal melanophores (Karne *et al.*, 1993), although it is not clear whether this is the amphibian variant of, for example, the mammalian ET_B receptor, or a distinct subtype of receptor. There is approximately 63% sequence homology between the ET_A and ET_B receptors and ~50% homology with the proposed ET_C receptor. In bovine cardiac sarcolemmal vesicles a protein binding endothelin-1 has been isolated but having a higher apparent molecular weight than that determined for ET_A, ET_B or ET_C, suggesting yet another possible receptor may be present in this tissue (Shannon & Hale, 1994), although this has not yet been fully characterised. It has been suggested that the identification of receptor subtypes is complicated by the internalisation of the receptor, rapid down regulation and desensitisation of some subtypes of receptor, and by the formation of ligand-receptor complexes of different dissociative kinetics depending on the ligand used (Bax & Saxena, 1994) .

However, there is other evidence for the existence of receptors not conforming to the typical pattern of ET_A or ET_B. For example, Sumner *et al.* (1992) described contraction of rat aorta to endothelin-3 which was more potently inhibited by the ET_A-selective antagonist BQ-123 than that to endothelin-1. Eglezos *et al.* (1993) again differentiated between receptors responsible for the endothelin-induced facilitation of twitch response in vas deferens (guinea pig) by use of BQ-123. The ET_A-selective antagonist had little effect on the endothelin-1 response, but inhibited the response to endothelin-3. Warner *et al.* (1993) found contractions of smooth muscle to endothelins could be divided according to their inhibition by different antagonists (BQ-123 and PD 142893), and suggested a subdivision of the ET_B receptor subtype (see section 1.3.1). Another study found the guinea pig gall

bladder to contain a receptor mediating contraction, atypical of the ET_A and ET_B subtypes (Battistini *et al.*, 1994). From these and a growing number of other examples, it is likely that more receptors will eventually be characterised.

The sequence of these receptors is unlikely to be homologous with those already discovered as no similar DNA has been found by low stringency probing of a cDNA library. However, as the requirements for endothelin binding to its receptors include only a site for the helical "head" section (ET_A) and a site for the linear C-terminus "tail" to bind (see above), these would be fulfilled if only sections of the extracellular domains were homologous.

1.3.1 Vascular and coronary receptors

Initial studies demonstrate that in vascular tissue, vasodilator ET_B receptors are located on the endothelium (Sakurai *et al.*, 1990). Stimulation of these may provoke release of local mediators (see section 1.4 below) producing vasodilatation which is not inhibited following administration of ET_A receptor antagonists (Fukuroda *et al.*, 1992). With this and other evidence, it is accepted that the transient vasodilator or hypotensive effect produced following endothelin administration is mediated by the ET_B receptor.

Vasoconstriction has been shown to be the result of stimulation of ET_A receptors on smooth muscle. Indeed, in human coronary artery and vein, Opgaard *et al.* (1994) found evidence of ET_A-mediated coronary constriction competitively inhibited by FR 139317. However this may not be the complete picture; in the anaesthetised rat, BQ-123 failed to inhibit completely the pressor response, suggesting that this is not the only vasoconstrictor receptor (McMurdo *et al.*, 1993). There is further evidence of vasoconstriction being mediated via ET_B receptors, despite initial failure to demonstrate the mRNA for the ET_B receptor in vascular smooth muscle (Sakurai *et al.*, 1990). The case for the expression of these receptors in vascular smooth muscle is complicated by the finding that in culture, the subtype expressed may change according to the culture conditions, ET_B predominating at late passage numbers whereas ET_A is predominant in cells at passages 10 to 15 (Eguchi *et al.*, 1994). This suggests that smooth muscle cells at least contain the potential for the expression of both ET_A and ET_B receptors. In functional studies, Clozel *et al.* (1992)

describe the vasoconstrictor effect, in rat, of the ET_B-selective ligand sarafotoxin 6c. In addition, Moreland *et al.*, (1992) have demonstrated the presence of vasoconstrictor ET_B receptors in venous smooth muscle. ET_B-mediated vasoconstriction has also been shown in human internal mammary artery and porcine coronary artery (Seo *et al.*, 1994) where no endothelial vasodilator ET_B receptors (linked to either NO or PGI_2), were found.

As well as the vasoconstriction mediated by ET_B receptors, there is evidence for another vasoconstrictor "$ET_{non\ A}$" receptor; in endothelium-denuded porcine coronary artery, vasoconstriction to endothelin-1, sarafotoxin 6b and endothelin-3 has been shown to be mediated by an ET_A-like receptor whereas endothelin-3 and sarafotoxin 6c activate a receptor which is not activated by endothelin-1 or sarafotoxin 6b, and hence has a profile unlike that of either ET_A or ET_B, (Harrison *et al.*, 1992). In the dog, sarafotoxin 6c caused reduced coronary flow despite the infusion of the ET_A antagonist BQ-123 (Teerlink *et al.*, 1994). In human vessels, Bax *et al.* (1994), indicate that the contraction of coronary artery is mediated by a subtype of ET_A receptor based on the different effectiveness of the antagonists BQ-123 and FR 139317, against contractions induced by endothelin-1 and sarafotoxin 6b.

From the evidence cited above, and further studies using different non-selective antagonists it is likely that the ET_B receptor is divided into two pharmacologically distinct subtypes These can be distinguished according to the differential selectivity of antagonists and according to the vascular site. The "classical" ET_B receptor which mediates vasodilatation and which is situated on the endothelium is sensitive to the non-selective endothelin receptor antagonist PD142893. This receptor has been provisionally termed ET_{B1}. The ET_B receptor which has been shown to mediate vasoconstriction in many preparations and is sited on vascular smooth muscle is PD142893-insensitive and has been provisionally termed ET_{B2} (Bax & Saxena, 1994; Douglas *et al.*, 1994). These subtypes have yet to be distinguished according to primary amino acid sequence.

It is likely that further work will reveal a wider range of endothelin/receptor interactions.

1.4 Actions and proposed roles of endothelins

Following discovery of the potent vasoconstrictor effect of the endothelins, a considerable amount of work was done to elucidate the role of these peptides in health and disease, but as yet, this has not been fully determined. Several studies performed measuring plasma levels of endothelin-1 by radioimmunoassay, in man gave ranges of 0.26 to 2.6 $pg.ml^{-1}$. Measurements in disease states in man showed higher concentrations of immunoreactive endothelin-1, in myocardial infarction (up to a 6.5x increase) and vasospastic angina pectoris (>2x increase). Other conditions where a rise was measured include uraemia and in patients undergoing haemodialysis (3.5x-6x increase), cerebral ischaemic conditions (4.4x-6x increase), pulmonary hypertension (2.4x increase) and hypertension of pregnancy (1.9x increase over a higher basal level in pregnant women); see Sokolovsky (1992) for review.

The role of endothelins in regulation of vascular tone and in multiple other physiological processes has been highlighted following measurement of their activity by bioassay. Studies of [^{125}I]-endothelin binding also show that the peptides may be active in a wide range of species and in many different preparations, and the growing use of antagonists, including those which are orally active, has led to further implication of the endothelins in pathophysiological processes. For example, Clozel *et al.* (1993) used the orally active ET_A and ET_B non-selective compound Ro 46-2005 in a variety of models and described its effectiveness against pathological vasospasm (renal and cerebral), as well as in a sodium-depletion model of hypertension.

As well as the vasoconstrictor and vasodilator activity of the endothelins previously described, the peptides have been shown to stimulate the proliferation of cells in culture (Battistini *et al.*, 1993; MacNulty *et al.*, 1990; Yamagishi *et al.*, 1993) including that of vascular smooth muscle cells. The mitogenic properties of endothelins are complex and are synergistic with other mitogens such as platelet-derived growth factor (see Battistini *et al.* (1993) for review).

The effect of these peptides also involves interactions with other vasoactive substances in several ways. Firstly the endothelins have been shown to modulate the release of substances

such as noradrenaline from perivascular nerve endings (Wong-Dusting *et al.*, 1989) probably via ET_B receptor stimulation as these have been identified on both adrenergic and cholinergic neurons (Takimoto *et al.*, 1993). The peptides have also been shown to release PGI_2 and NO from endothelial cells (Warner *et al.*, 1989; Emori *et al.*, 1991; Namiki *et al.*, 1992; White *et al.*, 1993), endothelium-derived hyperpolarising factor from pulmonary vasculature (Lippton *et al.*, 1991) and atrial natriuretic peptide from rat atria (Fukuda *et al.*, 1988). Secondly the endothelins have been shown to stimulate the enzymatic conversion of angiotensin I to angiotensin II in lung endothelium (Kawaguchi *et al.*, 1990). Thirdly, the effect of several vasoactive substances has been shown to be potentiated by the presence of sub-threshold concentrations of endothelin-1; Yang *et al.* (1990) demonstrated that both noradrenaline and 5-hydroxytryptamine responses, as well as the sensitivity of vessels to calcium chloride, were increased in the presence of endothelin-1 concentrations of up to 1nM, in human coronary and internal mammary vessels. A similar finding is reported in the contractile response to 5-hydroxytryptamine in porcine coronary artery (Nakayama *et al.*, 1991). In addition, the presence of monocytes or macrophages has been shown to increase the vasoconstrictor potency of endothelin-1 (Magazine *et al.*, 1994), an effect which has implications for the role of endothelins in inflammatory responses.

Some of the non-cardiovascular actions of the endothelin peptides are summarised in table 1.

Table 1:- Some actions of the endothelins (Adapted from Rubanyi & Polokoff, 1994)

Tissue	Receptor	Action
KIDNEY:		
Renal vasculature	ET_A/ET_B	Vasoconstriction (efferent>afferent)
Renal glomerulus	ET_A/ET_B	Reduction in glomerular filtration rate
		Mesangial cell proliferation
Juxtaglomerular apparatus	?	Reduction in renin release
Tubules/collecting ducts	?	Decrease in sodium reabsorption
LUNG		
Vasculature	ET_A/ET_B	Vasoconstriction (artery>vein); increased permeability
Airway epithelium	ET_A	Increased mucus and chloride secretion; prostanoid release
Airway smooth muscle	ET_A/ET_B	Contraction (enhanced with epithelial loss)
GASTROINTESTINAL TRACT:		
Stomach mucosa	ET_A	Ulcerogenicity, vasoconstriction
Ileum smooth muscle	ET_A/ET_B	Contraction and relaxation
Colonic mucosa	ET_A	Ion secretion
LIVER		
Hepatocytes	ET_A/ET_B	Glycogenolysis
		Contraction of bile cannaliculae
Portal vessels	ET_B	Vasoconstriction
URINARY TRACT	?	Contraction (bladder & urethra)

REPRODUCTION (female)		
Uterine & placental vasculature	ET_A/ET_B	Vasoconstriction
Endometrium	ET_A/ET_B	Prostanoid secretion stimulated
Myometrium	ET_A/ET_B	Stimulation of contractions
Breast	?	Mitogenesis
REPRODUCTION (male)		
Vas deferens	ET_A	Contraction
Testis	?	Steroid synthesis stimulated
BLOOD CELLS		
Platelets	?	Indirect inhibition of aggregation (via nitric oxide/prostacyclin production).
Polymorphonucleocytes	?	Stimulation of aggregation
Monocytes	?	Chemotaxis
EYE		
Retinal blood vessels	ET_A/ET_B	Vasoconstriction, mitogenesis (pericytes and endothelium)
Iris	ET_B	Modulation of pupil reaction and size
Ciliary body	?	Inhibition of aqueous humour production. reduction in intra-ocular pressure
BONE	?	Ostoblasts and osteoclasts - proliferation modulated

ENDOCRINE GLANDS		
Pituitary	ET_A/ET_B	ACTH release
	ET_C	Gonoadotrophin release
Thyroid	?	Inhibition of thyroglobulin secretion
Parathyroid	?	Modulation of parathormone secretion
Adrenal	ET_A/ET_B	Synergy with angiotensin II in stimulation of aldosterone secretion,
	$?ET_C$	Stimulation of catecholamine secretion
CENTRAL NERVOUS		Decrease in regional cerebral blood flow
SYSTEM		Inhibition of water intake
		Enhanced sympathetic efferent activity to kidney
	ET_A/ET_B	Elevation of plasma catecholamine, ACTH,
	$/ET_C$	arginine vasopressin, and glucose level
		Respiratory depression
		Increase / decrease in blood pressure
		Stimulation of pyramidal neurones
		Stimulation of pituitary glucose metabolism
		Substance P release

PERIPHERAL NERVES		
Motor and sensory nerves	?	Reduction in motor and sensory neuronal blood flow Decreased conductance Release of Substance P to activate spinal cord neurons
Parasympathetic system	?	Stimulation of acetylcholine (ACh) release, potentiation of ACh in intestine
Sympathetic system	?	Inhibition of noradrenaline release Potentiation of noradrenaline action Suppression / augmentation of baroreceptor reflexes

The cardiac effects and coronary effects of the endothelins are of particular relevance to this study and are described below.

1.4.1 Coronary effects of endothelins

Vascular effects of endothelins are of interest in the light of the vasospastic problems associated with coronary artery disease, and with reduced flow after reperfusion.

The endothelin-induced long-lasting vasoconstriction noted by Yanagisawa *et al.* (1988) is apparent in all vascular beds, but is often preceded by a transient vasodilator effect. This vasodilatation has been seen in several vascular beds but is not universally present. Its presence appears to be dependent on functional endothelium and is subject to tachyphylaxis (Le Monnier de Gouville & Cavero, 1991). In the coronary circulation also, the vasodilator / vasoconstrictor effects have been demonstrated in isolated perfused rat hearts (Baydoun *et al.*, 1990; Neubauer *et al.*, 1990), with differential sensitivities among microvessels (Homma *et al.*, 1992). The vasodilator component of the response in guinea pig hearts (Folta *et al.*, 1989) and in porcine coronary strips (Ushio-Fukai *et al.*, 1992) was shown to be endothelium dependent. The mechanism of this coronary vasodilatation has been shown

to vary between species; a study in rat heart found no evidence of PGI_2 involvement (Baydoun *et al.*, 1990) but in guinea pig heart (Karwatowska-Propopczuk & Wennmalm, 1990) and dog heart (Okamura *et al.*, 1992), PGI_2 dependence has been demonstrated .

The stimulation of endothelium to produce NO and PGI_2 has been linked with the vasodilator action of endothelins in several other preparations (Warner *et al.*, 1989; Mehta *et al.*, 1992; Namiki *et al.*, 1992). In studies using either endothelin-1 or endothelin-3 in coronary vessels, dependence on endothelial function appears to be species or preparation related; e.g. in dog coronary, the removal of endothelium does not result in reduction of the dilator phase of the response to endothelin-3 (Okamura *et al.*, 1992), whereas in porcine coronary artery, endothelium dependence and release of NO have been demonstrated (Pernow & Modin, 1993; Ushio-Fukai *et al.*, 1992). However, in cultured porcine coronary endothelial cells production of NO in response to endothelin-1 was not of major significance and PGI_2 production was predominant (Suzuki *et al.*, 1991). In this latter study, the presence of the appropriate receptors in endothelial cells in culture was not demonstrated and may be relevant to the effect seen (White *et al.*, 1993).

Vasoconstriction to higher doses / concentrations of endothelins in coronary vessels is consistently reported. This may be seen in isolated coronary preparations or in isolated heart preparations *in vitro*, (e.g. in rat, Baydoun *et al.*, 1989; Neubauer *et al.*, 1991) or following intracoronary injection of endothelins *in vivo* (e.g. in canine heart, Clozel & Clozel, 1989; Hom *et al.*, 1992). This reduction in coronary flow in canine hearts *in vivo* was selective for sub-epicardial vessels (Clozel & Clozel, 1989), suggesting that endothelin may be involved in local flow regulation in the heart.

Another vascular effect of the endothelins is the hypertrophic and hyperplastic growth of smooth muscle cells in culture, which may be of relevance to the development of atheromatous plaques in which muscle cell growth is a factor (Bobik *et al.*, 1990).

1.4.2 Other cardiac effects of endothelins

In addition to their coronary effects, endothelins have been shown to increase rate of beating of isolated atrial preparations (Ishikawa *et al.*, 1988). This was probably the result

of a direct effect on the cells. However, in the intact rabbit heart, an overall negative chronotropic effect has been reported following bolus injection of endothelins (Karwatowska-Propopczuk & Wennmalm, 1990). This could be an indirect result of the response to reduced coronary flow provoked by the peptides.

Endothelin-1 has been shown to be positively inotropic, increasing contractility of atrial or ventricular myocardium of rabbit, guinea pig and rat (Takanashi & Endoh, 1991; Hom *et al.*, 1992) although results in canine hearts are less clear (Takanashi & Endoh, 1991). Baydoun *et al.* (1989) reported an early positive inotropic followed by an irreversible negative inotropic effect in rat hearts after repeated bolus injection of endothelin in rat hearts; this could be a secondary effect of coronary vasoconstriction.

Another effect described for endothelin-1 is a hypertrophic action on rat myocardial cells. Evidence suggests that this is as a result of increasing transcription of specific genes such as those encoding α- and ß-myosin heavy chain (Wang *et al.*, 1992).

1.5 Intracellular calcium as a second messenger

1.5.1 Calcium and vascular tone

Changes in coronary vascular resistance are dependent on the tone of smooth muscle cells which surround the lumen of the vessel. Changes in this tone are important in maintenance of adequate coronary flow where the blood pressure may be unstable (Hoffman & Spaan, 1990). Ionised calcium is important in maintenance of tone and for contraction of the vascular smooth muscle cells, the interaction of calcium with contractile proteins being a major part of the development of tone. The calcium for contraction is therefore increased in local concentration in response to extracellular stimuli. It is, however, possible for contraction to occur without any discernible increase in intracellular calcium, and this could be due to a local change not being detectable within the overall cell calcium concentration, to increased sensitivity of contractile proteins to the calcium present (which may be modulated by DAG (diacylglycerol)), or to a direct phosphorylation of the contractile proteins themselves which may have a modulating effect (Morgan & Suematsu 1990). The contractile proteins actin and myosin overlap in the cells by an active process involving ATP and calcium in the manner outlined in figure I-5.

Briefly, three or four calcium ions are required to bind with a single molecule of calmodulin, an acidic protein within the smooth muscle cell, before this calcium-calmodulin complex will activate a molecule of myosin light chain kinase. This activated enzyme phosphorylates two light chains of myosin, by transference of a phosphate group from ATP. The phosphorylated myosin may then interact with the contractile protein actin by the formation and cycling of cross bridges, allowing sliding of one protein over another (see Bolton (1986) for review). Other structural proteins are involved in smooth muscle contraction. notably caldesmon which binds to filamentous actin in the absence of calcium (see Morgan & Suematsu, 1990) and may help to stabilise the actin-myosin complex and maintain a degree of smooth muscle contractile tone. Other kinases may also be involved in the phosphorylation of myosin light chain, which do not require an increase in intracellular free calcium ($[Ca^{2+}]_i$).

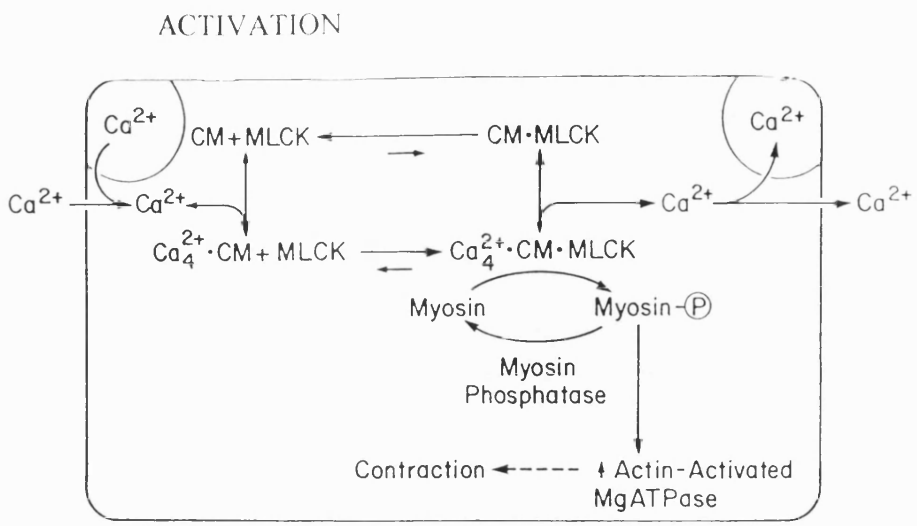


Figure I-5

Scheme for the biological regulation of myosin phosphorylation in smooth muscle cells. CM=calmodulin; MLCK=myosin light chain kinase; myosin-P = phosphorylated myosin. From Kamm & Stull (1985).

1.5.2 Calcium homeostasis

In order for this calcium-mediated effect to be controlled adequately, there must be constant regulation of the intracellular concentration of calcium ions, and mechanisms for an increase in calcium concentration when signalled by extracellular stimuli such as receptor activation. Under resting conditions, $[Ca^{2+}]_i$ in smooth muscle remains at nanomolar levels, 50-150nM concentrations of free calcium being regularly quoted. However, on stimulation this value has been shown to reach micromolar levels for a brief period. Following stimulation, the excess calcium is lowered following uptake into sarcoplasmic reticulum stores via a calcium calcium/magnesium ATPase or sequestered into organelles, or removed from the cell via extrusion mechanisms such as the calcium/magnesium ATPase and the sodium/calcium exchanger in the cell membrane (see Bolton, 1986).

1.5.3 Increases in intracellular calcium

The rise in intracellular calcium following interaction between a ligand and its receptor may be brought about via several linked mechanisms. The release of stored calcium is most commonly triggered by inositol (1,4,5) triphosphate (IP_3) acting on its receptor in the sarcoplasmic reticulum, this release from stores being responsible for the immediate transient $[Ca^{2+}]_i$ increase seen on stimulation. Another trigger is the activation of the so-called ryanodine receptor for which an endogenous sensitising ligand has been proposed (cyclic ADP ribose - Galione, 1992), and this is linked to a positive feedback of calcium-induced calcium release, via an action on the ryanodine receptor. This latter sarcoplasmic reticulum channel may also be opened by caffeine (Berridge, 1993).

1.5.4 Signal transduction for intracellular calcium increase

The intracellular events leading to release of IP_3 are usually evoked by a ligand acting on a GTP-dependent protein (G-protein)-linked receptor. The subsequent activation of phospholipase C ($ß_1$) results in the hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) giving the products IP_3 and DAG (see above). DAG may also activate protein kinase C (PKC) which is involved in the process of mitogenesis. PKC may also be involved in the amplification of the calcium signal through its phosphorylation of the L-type calcium channel. The activation of phospholipase C (γ_1) and subsequent IP_3 production, may also be brought about via activation of a tyrosine kinase-linked receptor, using ATP rather than GTP as a source of high energy phosphate. Following activation of a tyrosine kinase-linked receptor, other intracellular second messengers may be activated including phosphatidylinositol (PI) 3 kinase and mitogen activating protein (MAP) kinase which may both have a role in gene transcription and mitogenesis.

When intracellular stores in some cells are depleted, this has been shown to trigger the release of a small molecular weight messenger molecule (calcium influx factor; Randriamampita & Tsien, 1993), which provokes calcium influx through the cell membrane, allowing the calcium message to be prolonged.

As well as release of calcium from intracellular stores, the increase in $[Ca^{2+}]_i$ may arise following increased entry of calcium into the cell. This is secondary to activation of either receptor-operated calcium channels (ROCC) linked to specific receptors, or the activation of voltage-operated calcium channels (VOCC - predominantly L-type on smooth muscle) which respond to depolarisation of the cell membrane. These channels interact as described above, raising intracellular levels of calcium which may then be amplified by calcium-induced calcium release from sarcoplasmic reticulum. A further way in which these channels may interact with the other signalling apparatus is in the effect of PKC on the channel opening again potentiating the calcium signal.

1.6 Endothelin-stimulated signal transduction

There has been little distinction made between the second messenger systems employed by all three endothelins whether acting via ET_A or ET_B receptors, and so the intracellular effects of endothelins as a peptide group will be discussed together, unless it is indicated that a particular receptor subtype has been implicated in the response seen. The second messenger systems activated by the endothelins and which are described below, interact in a complex manner, and are still under investigation.

1.6.1 G-Proteins involved in transmembrane signalling by endothelins

Both the ET_A and ET_B receptors are of the conformation associated with G-protein interaction as stated above (section 1.3). In several studies, the effects of endothelins have been shown to be pertussis toxin sensitive, suggesting that the effect of the peptide is mediated via Gi or Go. In porcine coronary smooth muscle, for example, there is evidence that following receptor-ligand interaction the signal is transduced via such a G-protein, leading to opening of VOCC either directly or indirectly (Kasuya *et al.*, 1992). In rabbit rectosigmoid smooth muscle cells, endothelin receptors were found to be coupled to two subtypes of the Gi protein, each with a different signalling outcome (Bitar *et al.*, 1992). In porcine endothelial cells in situ, a pertussis toxin-sensitive mechanism for the elevation of intracellular calcium via calcium influx, has been described (Aoki *et al.*, 1994), and in guinea pig atrial myocytes a pertussis toxin sensitive G-protein (probably Gi) has been implicated in cardiac inhibition by endothelin acting on ET_A receptors, reducing cyclic AMP formation (Ono *et al.*, 1994).

However, some transduction events in response to endothelins are pertussis toxin-insensitive (Bobik *et al.*, 1990), hence it is possible that some events are either mediated through different G-proteins or not G-protein-linked at all. In another tissue (rat liver, Jouneaux *et al.*, 1994), ET_B receptors have been shown to be coupled to Gs and Gq, suggesting tissue specificity of the transmembrane coupling.

1.6.2 Calcium signalling

Endothelin was initially postulated as an endogenous ligand for L-type voltage operated calcium channels, as antagonists to these channels reduced the effect of endothelin (Yanagisawa *et al.*, 1988). However, though chelation of extracellular calcium removed contractile responses in rat aortic strips, the response was not totally abolished using a dihydropyridine ((+)PN200-110). The effect on the ion channel was shown to be indirect, as no displacement of binding of L-type calcium channel ligands by endothelin was seen (van Renterghem *et al.*, 1988). One later report suggests that the binding of tritiated nicardipine to calcium channels in the renal artery may be displaced by endothelin-1 (Amenta *et al.*, 1993) but the significance of this, and its relevance to other calcium channel ligands, need further clarification. Many further studies have shown that endothelins do indeed produce an increase in intracellular calcium and this has been demonstrated by functional studies and calcium imaging techniques as well as by voltage-clamping studies (van Renterghem *et al.*, 1988).

The increase in $[Ca^{2+}]_i$ in response to endothelins is initiated via several different pathways. Tritiated IP_3 metabolism has been shown to increase as a result of endothelin stimulation of smooth muscle (van Renterghem *et al.*, 1988) leading to a transient increase in $[Ca^{2+}]_i$, and this response has since been demonstrated in many different cell types and preparations. For example, in human atrial myocytes (Vogelsang *et al.*, 1993), fibroblasts (MacNulty *et al.*, 1990) and in endothelial cells (Emori *et al.*, 1991), a rise in IP_3 has been shown to follow endothelin-1 stimulation. Other receptors on the sarcoplasmic reticulum may also be activated following endothelin stimulation, including the ryanodine receptor (Wagner Mann *et al.*, 1992; Wagner Mann & Sturek 1992), again resulting in the release of calcium from stores. More detail on the actions of endothelin in increasing $[Ca^{2+}]_i$ in smooth muscle cells is included below (section 1.7), as this may be of particular relevance to the development of vasospasm in coronary heart disease.

1.6.3 Activation of other ion channels

1.6.3.1 Cation channels

Van Renterghem *et al.*, (1988) also describe effects of endothelin on membrane potential in a rat smooth muscle cell line, comprising a transient hyperpolarisation followed by depolarisation with calcium spiking. These phases corresponded with an outward K^+ current, followed by influx of cations probably via a non-selective cation channel which is permeable to Ca^{2+}. This early study suggested that the depolarisation allowed membrane potential to reach the threshold for L-type channels to be activated. Kasuya *et al.*, (1992) argue that depolarisation alone, however, is insufficient to explain the degree of calcium channel activation by endothelin-1, hence a receptor operated mechanism may also be involved. A link has been demonstrated between increases in $[Ca^{2+}]_i$ and $[Na^+]_i$ in rat vascular smooth muscle cells (Okada *et al.*, 1991), the uptake of both of these ions being stimulated by endothelin-1.

1.6.3.2 Chloride channels

Opening of the chloride channels in smooth muscle leads to depolarisation as the chloride reversal potential is less polarised than the resting potential in this tissue. Mesangial cells are closely related to smooth muscle cells, and retain many of their features. In experiments in mesangial cells, there is evidence that the ET_B receptor is linked to depolarisation via the opening of chloride channels and the closure of a potassium channel (Hu *et al.*, 1993). It has been reported that endothelin also induces depolarisation in smooth muscle cells by the opening of a chloride channel which is calcium activated (Iijima *et al.*, 1991). Depolarisation also would potentiate the existing calcium signal by allowing further calcium entry through voltage operated channels. The overall importance of this mechanism is not clear, but is another mechanism of potentiation of the endothelin effect which adds to its overall potency. In cardiomyocytes, however, the chloride conductance has been shown to be inhibited by endothelin-1, the mechanism being ET_A mediated, and pertussis toxin-sensitive (James *et al.*, 1993).

1.6.3.3 Potassium channels

It has been reported that endothelins block the ATP-sensitive potassium channels in porcine coronary smooth muscle and initiate depolarisation in this way (Miyoshi *et al.*, 1992). These events lead to an increase in vascular tone. This increase in tone may, though, be alleviated in turn by the production of endothelium-derived hyperpolarising factor which opens potassium channels in smooth muscle as described in rat coronary vascular beds by Sakuma *et al.* (1993).

In the heart, the potassium channel effects are complex. Ono *et al.*, (1994) describe hyperpolarisation of cardiac cell membranes by ET_A receptor-mediated stimulation of an outward potassium current, and subsequent effects included inhibition of inward calcium current. In contrast, PKC-mediated potassium channel inhibition has been reported to result in the potentiation of calcium currents by endothelins in rat cardiac myocytes (Damron *et al.*, 1993).

1.6.4 PKC activation

PKC effector mechanisms are complex involving feedback on calcium entry (see above), and the enzyme is also involved in the activation of the Na^+/H^+ exchanger which is reported to result in intracellular alkalinisation. PKC activation has been demonstrated as a result of endothelin-receptor interaction in vascular smooth muscle cells, the effects of endothelin on intracellular pH being blocked by staurosporine, a PKC inhibitor (Danthuluri & Brock 1990). This pH rise may be responsible for sensitisation of the contractile proteins to calcium, allowing contraction without a discernible rise in $[Ca^{2-}]_i$. The sensitisation of the contractile apparatus following endothelin receptor activation has been shown also in strips of rat aorta, where sarafotoxin 6b initiated a contraction apparently independent of release of calcium from stores in calcium-free buffer. This effect was sensitive to the PKC inhibitor H-7 (Watanabe *et al.*, 1993). Endothelin-1-induced activation of the Na^+/H^+ exchange has also been reported in rat mesangial cells and rat ventricular myocytes. The mechanism identified again includes an increase in intracellular alkalinisation (Simonson *et al.*, 1989;

Krämer *et al.*, 1991), resulting in increased sensitivity to calcium of the contractile proteins in myocytes. In mesangial cell experiments, the increase in PKC activity on endothelin stimulation was accompanied by stimulated mitogenesis (Simonson *et al.*, 1989). The adverse effects of endothelins on contraction and intracellular metabolism after ischaemia / reperfusion may be partly attributed to the activation of Na^+/H^+ exchange as these have been shown to be reversed by methylisobutyl amiloride, which inhibits this mechanism (Khandoudi *et al.*, 1994).

The activation of the Na^+/H^+ exchange mechanism by endothelins may be independent of PKC activation, in response to the peptide binding to some subtypes of the endothelin receptors, a possible means of differentiation between the subtypes of receptor on functional grounds (Frelin *et al.*, 1991).

The measurement of pH generally incurs use of fluorescent markers such as BCECF, which are usually used in a HEPES-buffered medium. The effects of endothelins in physiologically buffered systems are not so clearly documented. In human umbilical artery smooth muscle cells, however, no effect from endothelin on intracellular pH was seen, using BCECF in a HEPES-buffered medium (Gardner *et al.*, 1992). This may suggest that the effect of endothelin on the Na^+/H^+ exchanger is cell or species dependent, in addition to other considerations.

1.6.5 Phospholipase activation

As well as the activation of phospholipase C, which results in IP_3 production, as discussed above (section 1.5.4), the interaction of endothelins with their receptors initiates enzyme activity in other phospholipases. These include phospholipase D (PLD) (Plevin *et al.*, 1994), which, through its hydrolysis of phosphatidyl choline, releases phosphatidic acid, which in turn may have second messenger roles in the contraction of smooth muscle. The further hydrolysis of phosphatidic acid (by phosphatidic hydrolase) to diacylglycerol may indicate that the activation of PKC by this route may also be a method by which endothelins are involved in the calcium message and cell growth. It has been shown that the endothelin-stimulated effect of PLD may be itself regulated by PKC in hypoxic smooth muscle cells

(Plevin *et al.*, 1994). The interaction between PKC and PLD in other cells has been demonstrated, where PLD activation by endothelins has been shown to be by both PKC-dependent and -independent pathways (rat fibroblast and glioma cell lines - Ambar & Sokolovsky 1993; rat mesangial cells - Kester *et al.*, 1992). It has also been shown that PLD activation is dependent on the action of tyrosine kinase (see below), in a rat aortic smooth muscle cell line (Wilkes & Boarder, 1992).

The activation of phospholipase A_2 (PLA_2) by endothelins has been reported, probably by direct coupling of the receptor to the enzyme in renal interstitial cells, and resulting in an increase in prostaglandin E_2 production (Barnett *et al.*, 1994). This activation may also be independent of PKC.

1.6.6 Tyrosine kinase activation

Endothelins have been shown to stimulate the phosphorylation on tyrosine in several proteins. MAP kinase p42 phosphorylation on both tyrosine and threonine has been shown in mesangial cells to follow endothelin-1 stimulation, and involvement of both tyrosine kinase and PKC has been implicated (Wang *et al.*, 1993). In osteoblast-like cells, DNA synthesis stimulated by endothelin-1 has been associated with an increase of tyrosine phosphorylation, in a manner synergistic with platelet-derived growth factor (PDGF) (Schvartz *et al.*, 1992). This parallels the synergy of endothelins with PDGF and other mitogens described by Battistini *et al.*, (1993). In (rat aortic) vascular smooth muscle cells, tyrosine kinase activation in response to endothelin-1 has also been demonstrated (Koide *et al.*, 1992a,b). However, it was not considered that this was sufficient to account for stimulation of muscle cell proliferation, as increased DNA synthesis and increase in cell number did not accompany the phosphorylation seen (Koide *et al.*, 1992a). In view of the finding that the same set of proteins was also phosphorylated on tyrosine by PDGF, this indicates that synergistic interaction of endothelin with other growth factors may be necessary in vascular smooth muscle cell proliferation. It is possible also that tyrosine kinase activation in vascular smooth muscle may not be associated with cell proliferation, but with other responses such as contraction. It has been demonstrated, however, that

DNA synthesis may be stimulated in similarly cultured vascular smooth muscle cells in response to endothelin, the effect being mediated via ET_A receptors (Eguchi *et al.*, 1992).

1.7 Models of measurement of intracellular calcium in smooth muscle cells

1.7.1 Calcium indicators

Intracellular calcium may be measured using a generation of fluorescent calcium indicators which were developed for their ability to bind to calcium with high affinity, and for their fluorescent qualities. Many are analogues of the calcium chelating agent BAPTA (1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid, a double aromatic analogue of EGTA) which are highly selective for calcium (Grynkiewicz *et al.*, 1985). Compounds such as quin2 are not fluorescent unless calcium is bound. Fura-2 is a compound fluorescent at a 380nm excitation wavelength, which fluoresces in response to a different excitation wavelength (340nm) when calcium binding occurs, allowing calculation of the ratio of bound to unbound fura-2. The advantage of this is that differences in total fluorescence related to the local concentration of the indicator are accounted for. Indo-1 also has the advantage of allowing the bound:unbound ratio to be calculated but, in this case, the excitation wavelength remains the same whereas the emission from the two forms of the indicator occurs at different wavelengths (Cobbold & Rink, 1987).

1.7.2 Fluorimetric assay of calcium responses to endothelins in vascular smooth muscle cells

Many studies on vascular smooth muscle cells have been performed using endothelins, giving a variety of different results, some showing a plateau phase of calcium elevation after an initial spike, and others with only the transient which has been attributed to IP_3-induced mobilisation of calcium from intracellular stores. The variability has been attributed to differing effects of high and low doses of endothelins as well as to the multiplicity of models used in measurement of $[Ca^{2+}]_i$ (Wagner Mann & Sturek, 1992). Species differences as well as differences in the manner of cell preparation and observation are possible reasons for the variation of responses described. Some of the models which have been used are outlined below, together with examples of the experimental findings from their use.

1.7.2.1 Single cell studies

Responses in fresh enzymatically-dispersed porcine coronary smooth muscle cells were described by Wagner Mann & Sturek (1991). These authors suggest that a freshly dispersed single smooth muscle cell more closely approaches physiological reality than, for example cultured cell preparations. From using a combination of endothelin treatment with caffeine-depletion of intracellular stores and potassium-induced depolarisation they conclude that the stores from which calcium is released are different in porcine preparations than in bovine smooth muscle cells. The stores in porcine cells are described as less ryanodine sensitive than those in their bovine equivalent (Wagner Mann *et al.*, 1991). Both peak and plateau phases of the response are described as inclusive of calcium influx. In porcine cells, however, no plateau phase of sustained calcium rise was evident when exposure of the single cells to the peptide was prolonged (Wagner Mann & Sturek, 1991), superfusion of the cells being included in the protocol in experiments where the plateau was demonstrated. This is attributed to depletion of intracellular stores with time, and the net calcium influx being balanced by efflux.

In single cells from rat aortic smooth muscle cell cultures also, a biphasic calcium increase has been demonstrated (Iijima *et al.*, 1991). The sustained phase was virtually abolished in the absence of extracellular calcium. The efflux of chloride ions, through chloride channel activation, and subsequent depolarisation was cited in this study as responsible for the plateau phase of the increase in $[Ca^{2+}]_i$.

It can be argued that the detail of the calcium response in a single cell can more clearly be described than in a larger population.

1.7.2.2 Cell populations in suspension

Calcium measurements from cells in suspension give the mean response of a larger number of cells, allowing for responses in small numbers of atypical cells to be ignored. Disadvantages include the excessive numbers required in a single experiment, and the loss of responses in individual cells such as the spiking of calcium transients. The disruption of a growing monolayer for dispersal may also result in some damage to cells.

Van Renterghem *et al.*, (1988) measured intracellular calcium rise in a rat vascular smooth muscle cell line (suspensions of A7r5 cells) by flow cytometry. The increase in $[Ca^{2+}]_i$ could be explained mainly by a mechanism of IP_3-mediated release, rather than entry through calcium channels. A sustained phase of calcium elevation was not evident in these experiments.

Wallnöfer *et al.*, (1989) also performed some of the earlier fluorimetric assays to determine whether endothelin acted as an endogenous VOCC activator. They concluded that this is not the case but that their results were compatible with endothelin activation of a receptor-operated calcium channel (ROCC). In a suspension of cells from a rat aortic cell line (A10), a peak and plateau response to endothelin were seen, the peak and plateau both being susceptible to removal of extracellular calcium, although the initial component of the response was not completely lost in calcium-free conditions. Parallel experiments revealed that the sustained intracellular calcium increase was greater than could be explained by entry alone, as measured by .^{45}Ca influx.

Ihara *et al.*, (1991) describe the calcium responses in porcine coronary artery smooth muscle cells in culture to endothelin-1 and endothelin-3. Endothelin-1 initiated an increase in $[Ca^{2+}]_i$ whereas endothelin-3 had little effect compared to endothelin-1. Parallel binding studies revealed no ET_B-like receptors (with potency of ET-1=ET-3) on the cultured cells, although on cell membranes from the original endothelium-denuded porcine coronary arteries two binding sites were found, one endothelin-1-selective (ET_A) and the other resembling ET_B in its potency profile. The contraction of coronary arteries to both endothelins was demonstrated leaving the receptor subtype involved in question. It is possible that cells containing ET_B receptors are not present after cell culture, or that this subtype is downregulated under some culture conditions.

1.7.2.3 Cell populations in confluent monolayers

Cells grown in this manner have the capability of intercellular communication, and could be argued to be more likely to act as a physiological unity in response. Smooth muscle cells

which grow in culture in this manner do not require dispersion. However, some of the detail of an individual cell's response will be lost.

The requirement of calcium for both phases of the calcium response was demonstrated by Gardner *et al.*, (1992) in human umbilical artery smooth muscle cells grown to confluence on coverslips. Low extracellular calcium, as well as nicardipine, attenuated both the immediate spike and sustained phases. The presence and involvement of VOCCs in the endothelin-1 response, was demonstrated using the fluorescence-quenching properties of Mn^{2+} which enters via these channels in depolarised cells.

Okishio *et al.*, (1992) demonstrated a peak and plateau phase of the response to endothelin-1 in rat aortic smooth muscle cells grown in monolayers on coverslips. The peak and plateau phases were sensitive to addition of S-nitrosothiols, NO-donating compounds, with the plateau most severely attenuated. The authors suggest that the calcium influx component of the rise in $[Ca^{2+}]_i$ is thus affected by these compounds, but not release from stores.

Using rat aortic smooth muscle cells grown in monolayers, Little *et al.*, (1992) compared responses in $[Ca^{2+}]_i$ following addition of endothelin-1 and endothelin-3. A sustained rise in $[Ca^{2+}]_i$ is not clearly indicated and is not discussed. The endothelin-1- and endothelin-3-stimulated calcium transient was reduced in the absence of extracellular calcium. The transient was described as being due to mobilisation of intracellular stores as well as to entry through calcium channels. IP_3 production was also determined by chromatographic measurement of the tritiated compound. The results indicate that mechanisms of calcium increase differ between the two endothelins, although mobilisation of intracellular stores of calcium was a feature of the response to both. IP_3 was not increased by addition of endothelin-3 to a level which would mobilise calcium, whereas endothelin-1 was responsible for a greater increase in production above basal levels. The authors postulate that cADP ribose could release the intracellular calcium stores. There is no evidence to suggest which receptor subtype is involved in the response to either peptide.

1.7.2.4 Front surface fluorimetry

This technique involves more specialised equipment, but measures $[Ca^{2+}]_i$ in cells *in situ*. Thus the findings from these studies should more closely resemble the physiological state and in some studies, the calcium measurement can be performed simultaneously with measurement of developed force. Yoshida *et al.*, (1994) performed such studies in endothelium-denuded rabbit mesenteric artery. In these smooth muscle cells, the contractile responses were shown to be mediated by ET_A receptors as BQ-123 was completely inhibitory and the ET_B receptor agonist IRL 1620 was ineffective. The calcium responses were shown to be due to influx at low endothelin concentrations, as there was no increase in $[Ca^{2+}]_i$ to these concentration in calcium-free extracellular solution. Higher concentrations did result in mobilisation of calcium from stores. The increase in force in the strips was out of proportion to the rise of $[Ca^{2+}]_i$, when compared with the contractions produced by noradrenaline and by high potassium. In permeabilised muscle strips, increasing extracellular calcium concentrations caused a simultaneous increase in $[Ca^{2+}]_i$ and in force development. Endothelin-1 had little effect on $[Ca^{2+}]_i$ but very markedly increased force, suggesting that the peptide increased the sensitivity of the contractile proteins. BQ-123 inhibited the endothelin-1-induced enhancement of this calcium-induced contraction suggesting that the protein sensitisation is mediated via ET_A receptors.

The question remains of the contribution of the choice of model to the results gained. Whether the same cell type grown under identical culture conditions will respond to endothelin in the same way if measurements are made in a cell suspension, in monolayers of confluent cells or in single cells, is not clear.

1.8 Role of endothelins in ischaemia and reperfusion

There are several indications of a potential role for the endothelins in the cardiac damage which follows ischaemia and/or reperfusion (see section 1.1 above), most of which mirror the evidence of the involvement of these peptides in ischaemic damage of other organs, such as the kidney and brain (see Simonson, 1993). Direct injection of endothelin-1 into dog coronary arteries results in coronary vasospasm and prolonged myocardial ischaemia (e.g. Kurihara *et al.*, 1989). The initial evidence for the endothelins' involvement in ischaemic heart disease was for an increase in cardiac and plasma concentrations of immunoreactive endothelin after myocardial infarction (Watanabe *et al.*, 1990) and possibly following coronary vasospasm and percutaneous transluminal coronary angioplasty, although there is conflicting evidence in the latter case (see Tønessen *et al.*, 1993). These increased levels probably result from an increase in *de novo* synthesis of the peptides, as there is no evidence of vesicular storage of endothelins. Indeed there are reports in several models of an increase in gene expression and subsequent release of endothelin-1 from the heart after ischaemia / reperfusion. In one study in porcine hearts, reperfusion after 10 minutes of coronary arterial occlusion resulted in an increase of released endothelin-1 (Tønessen *et al.*, 1993). A separate study in a porcine model suggested the endothelium as source of the increased endothelin levels released on reperfusion (Franco-Cereceda *et al.*, 1994). The endothelial cells may not be the source of the increase in endothelin synthesis, however. In another report, porcine cardiomyocytes biopsied from animals undergoing coronary ligation were found to contain increased endothelin-1 mRNA on Northern blotting, compared with controls, although there was no increase in coronary vascular mRNA (endothelial or smooth muscle; Tønessen *et al.*, 1995). In the isolated rat heart model also, an increase in endothelin release was seen in early reperfusion following low-flow hypoxic ischaemia, which could adversely affect the coronary vasculature although it is suggested (Brunner *et al.*, 1992) that the concentrations reached are too low to have an effect on myocardial contractile function (stunning).

In line with the increase in local accumulation of endothelins after ischaemia / reperfusion, there are several reports relating to the involvement of endothelins in subsequent cardiac damage. The coronary vasospasm which follows experimental balloon-induced arterial injury in pigs can be significantly reduced after intracoronary injection of the ET_A antagonist BQ-123 (Fukai *et al.*, 1993), suggesting potential involvement of the peptides in vasospasm after percutaneous transluminal coronary angioplasty. Involvement in the development of myocardial infarction is also suggested; a study by Watanabe *et al.*, (1991) showed that anti-endothelin antibodies could reduce the size of myocardial infarction in the rat. This potential involvement of the peptides in myocardial pathology is further supported by a study in which BQ-123 reduced infarct size in the dog heart by up to 40% (Grover *et al.*, 1993). However, this projected (ET_A receptor-mediated) involvement of endothelins in exacerbating the size of myocardial infarction may not be common to all species, as McMurdo *et al.*, (1994) found no reduction in infarct size in the rabbit after infusion of the ET_A receptor antagonist FR 139317. Conversely, another report suggests that pathological concentrations of endothelins following coronary occlusion may have positive effects on collateral flow; in a study in dogs (Donckier *et al.*, 1994) injection of endothelin-1 appears to have caused a selective increase in collateral blood flow to the ischaemic region without resulting in changes in flow to the non-ischaemic region. The increased flow, however, did not ameliorate recovery of function which was indeed exacerbated in endothelin-treated hearts. The mechanism of this loss of cardiomyocyte function may be related to increased calcium toxicity; endothelin-1 has been shown to cause increased cellular damage in simulated ischaemia in myocytes, through an effect blockable by use of nifedipine (Stawski *et al.*, 1991).

The damage resulting from endothelin administration may not be completely due to a direct action of the peptides. For example, a role for blood cells in the damage in which endothelin is implicated has been postulated. López Farré *et al.* (1993) describe endothelin-stimulated neutrophil adhesion in the rabbit heart *in vivo* and *in vitro*, via increased expression of integrin adhesion molecules (CD18 and CD11b complex). Increased accumulation of neutrophils and their release of superoxide and other reactive species could

contribute to the vascular damage leading to increased development of infarct size and other postulated detrimental effect of endothelins. It is also likely that the ST segment elevation of the electrocardiogram (an indicator of myocardial injury) and capillary leakage resulting from endothelin-1 administration may be aggravated by its release of other local mediators (Filep *et al.*, 1994) such as thromboxane A_2 and platelet activating factor, via activation of ET_A receptors. Another study in isolated canine blood vessels provides evidence against hypoxic vasoconstriction being mediated by endothelin-1, however, as BQ-123 was ineffective against its development (Douglas *et al.*, 1993).

In order to investigate further the background for the involvement of endothelins in myocardial infarction, Liu *et al.*, (1990) explored the circumstances in which [^{125}I]-endothelin-1 binding to rat cardiac membranes was increased, selecting cardiac acidosis, hypoxia, reoxygenation and ischaemia / reperfusion or recurrent ischaemic episodes. These experiments were performed in isolated perfused rat hearts under conditions of constant flow using modified Krebs perfusate. It was found that 30 minutes global ischaemia alone, 30 minutes of ischaemia with reperfusion, and reoxygenation after 30 minutes of hypoxia (but not hypoxia alone) resulted in a significant increase in B_{max} for endothelin-1. The mechanism of this increase in binding site density was also investigated by separation of membrane fractions and evidence was found of increased externalisation of receptors (Liu *et al.*, 1989), and it was suggested that these were probably of the ET_A receptor subtype. An increase in cardiac membrane binding (B_{max}) of endothelin-1 was also seen in isolated perfused rabbit hearts (Fushimi *et al.*, 1992) under a similar protocol. The time-dependent increase in B_{max} of endothelin-1 binding can be attenuated by pretreatment with amlodipine in rat hearts in a dose-dependent manner (Nayler *et al.*, 1992) or by L-nitro-arginine in rabbit hearts (Saito *et al.*, 1993).

With the above evidence of increased binding site density, the question needs to be addressed of whether this represents an increase in functional receptors for endothelin-1, accompanied by an increase in coronary or myocardial endothelin reactivity to the peptides. In dog coronary arteries, a report by Fushimi *et al.*, (1992) describes increased reactivity,

selective to endothelin-1, following 60 minutes of ischaemia and 120 minutes of reperfusion. It was suggested that the increased reactivity could be mediated by polymorphonuclear leucocytes (PMNs) as when *ex vivo* preparations of dog coronary were subjected to simulated ischaemia and reperfusion, it was necessary for activated PMNs to be present for the enhancement of reactivity to be seen. This potentiation would not necessitate the presence of an increased number of functional contractile receptors, but in a later study in rabbit heart, upregulation of binding sites does appear itself to be potentiated by the presence of PMNs (Saito *et al.*, 1993). McMurdo *et al.*, (1991) describe an increase in the duration of coronary constriction to a single (100pmol) dose of endothelin-1 in the isolated perfused rat heart, although the magnitude of the response was not affected. In the isolated rat hearts in this study, the conditions under which the increase in endothelin-1 binding density were found to be increased are reproduced. Similar experiments in rat hearts were performed by Neubauer *et al.*, (1991) measuring changes in coronary flow rate to successive bolus doses of endothelin-1 at constant perfusion pressure. The contractile responses to endothelin-1 after 30 minutes of ischaemia with subsequent reperfusion, were found to be significantly enhanced but the enhancement was accompanied by loss of the initial vasodilator component of the response described by Baydoun *et al.*, (1990). The same authors also attempted to investigate the effects of hypoxia and reoxygenation on responses to endothelin-1. However, it was impossible to reproduce the severity of the hypoxic conditions used in Liu's study, with the result of increased endothelin binding without loss of steady state; hence these conditions were modified to allow measurements to be made. The lack of enhancement of the vasoconstrictor endothelin-1 response after reoxygenation must be considered in the light of these modifications, as no binding studies were performed under the new conditions.

The above evidence is certainly suggestive of an increase in coronary reactivity following ischaemia and reperfusion, which correlates with the increase in binding site density and putative receptor number. However, the contribution made by loss of a physiologically opposing vasodilatation after reperfusion is not clear.

1.8.1 Models of ischaemia and reperfusion

The model of ischaemia / reperfusion used by Liu *et al.*, (1990) and later by others to determine whether the upregulation of binding sites resulted in an increased functional effect for the endothelins, is the Langendorff heart preparation. In this model, the coronary arteries in the isolated heart are perfused by retrograde perfusion of the aorta. Perfusion is achieved using an oxygenated, physiologically balanced and pH-buffered solution of salts, commonly Kreb's Henseleit solution. The heart may be perfused either at constant pressure, measuring the changes in flow rate as utilised by Neubauer *et al.*, (1991), or at a constant flow rate, measuring changes in perfusion pressure, as utilised by McMurdo *et al.*, (1991). These parameters may both represent changes in coronary radius, as demonstrated by use of derivations of the Hagen-Poiseuille equation:-

$$\frac{\text{Pressure}}{\text{Flow}} = \frac{1}{r^4} \times \frac{l \cdot \eta \cdot 8}{\pi}$$

Where r is radius of the vessel, l is the length of the tube system, and η is the fluid viscosity. The viscosity of the perfusate and length of the system remain constant under experimental protocol, so the equation may be simplified as follows:-

$$\frac{\text{Pressure}}{\text{Flow}} \sim \frac{1}{r^4}$$

As either pressure or flow is constant this will allow further simplification, allowing measurement of the variable parameter to reflect changes in vessel radius.

The isolated perfused heart model cannot replicate exactly the conditions of physiological perfusion, as no perfusion system exactly mimics the fluctuations of perfusion pressure provoked by systole and diastole of the normal heart *in vivo*. Moreover, myocardial contraction compresses the coronary arteries cyclically, and a mean value of the pressure or flow must be taken (Hoffman & Spaan, 1990), which also may overlook differences in vascular resistance across different areas of the myocardium.

Ischaemia is the insufficiency of blood flow to meet the demands of the affected area, hence the reduction or removal of flow of a perfusate should not strictly be termed ischaemia. However, for simplicity, the term ischaemia will be used below to describe both situations. Simulation of ischaemia may be achieved by stopping flow to the heart completely, (global zero-flow ischaemia), or by occlusion of a vessel supplying part of the myocardium. A low-flow ischaemia may also be used to mimic more closely the pathological processes which are sequelae of coronary narrowing. In the binding experiments performed by Liu *et al.*, (1991), a global model of zero-flow simulated ischaemia was used to produce an upregulation of endothelin-1 binding sites. Hence this protocol will be followed in the experiments described below.

1.9 Aims

1.9.1 Ischaemia / reperfusion experiments

In view of the changes in binding density of endothelin-1 after 30 minutes of global zero flow ischaemia in rat hearts (Liu *et al.*, 1990) and the reported increase of effect of endothelin-1 (Neubauer *et al.*, 1991; McMurdo *et al.*, 1991):

To investigate whether the coronary responsiveness to all three endothelin peptides and to an ET_B-selective agonist (sarafotoxin 6c) is similarly increased after a similar period of ischaemia with reperfusion.

To determine whether vasodilator loss is a feature of this protocol by using a range of vasodilator agents with different mechanisms of action.

To evaluate the contribution of vasodilator loss to the increase in contractile response.

To determine the selectivity of the vasoconstrictor enhancement by use of other vasoconstrictors.

To evaluate which receptor subtypes are responsible for the components of the coronary response to endothelins in this model, by investigation of the effect of BQ-123.

1.9.2 Calcium experiments

In view of the atypical endothelin receptor subtypes found by Harrison *et al.*, (1992) to be involved in contraction of porcine coronary arteries:

To investigate the receptor subtype involved in the intracellular increase in free calcium in porcine coronary artery smooth muscle cells in response to endothelin by use of the antagonist BQ-123.

With regard to the differences in the form of the $[Ca^{2+}]_i$ response in smooth muscle cell preparations as described by Wagner Mann & Sturek (1991):

To evaluate the manner in which the intracellular calcium responses to endothelins are dependent upon the preparation of vascular smooth muscle cells and the model used.

With regard to the tyrosine phosphorylation seen in response to endothelins in smooth muscle described by Koide *et al.*, 1992) and the complex interactions of the endothelin-induced signal transduction mechanisms:

To determine whether there is a contribution from tyrosine kinase activation to the calcium response in vascular smooth muscle cells.

METHODS

2.0 Coronary reactivity in rat hearts

2.1 Modified Langendorff perfusion technique

Materials and solutions (see section 2.7)

Perfusate: Modified Krebs Henseleit Solution

Composition (mM):

NaCl	118
KH_2PO_4	1.2
$CaCl_2$	1.23
$NaHCO_3$	25
D-Glucose	11.6
$MgSO_4$	1.2
KCl	4.7 (or 2.0 where indicated)

The perfusate was gassed using 95% O_2: 5% CO_2 and warmed to 37°C using a heating coil in the perfusion circuit. Following gassing, pH was within physiological limits (7.35 - 7.45).

Methods

Male Wistar rats weighing 250-350g were anaesthetised using sodium pentobarbitone (Sagatal 75mg.Kg^{-1}) injected intraperitoneally (i.p.). Following sacrifice by cervical dislocation, hearts were rapidly excised and immersed in ice-cold Krebs Henseleit solution, before being perfused via the aorta, at a constant flow rate of 10ml.min^{-1} with warmed, oxygenated perfusate. The heart was maintained at 37°C using a heating jacket. Thebesian vein effluent was removed via a 25G needle inserted into the left ventricle.

Coronary perfusion pressure was measured via a pressure transducer attached to the aortic cannula and developed tension measured under 2g resting tension by an isometric transducer attached to the apex of the left ventricle. This apical deflection was used to

trigger a heart rate meter. All parameters were recorded using a Devices chart recorder or a Grass Polygraph.

Each preparation was allowed to equilibrate for a minimum of 20 minutes prior to commencement of the experimental protocol. Experiments were performed with appropriate time-matched controls and in parallel where possible.

2.2 Ischaemia / reperfusion experiments

Following equilibration, 30 minutes of global zero-flow "ischaemia" was achieved by cessation of perfusion. Superfusion was commenced (at 10ml.min^{-1}) to avoid cooling, using deoxygenated (95% N_2: 5% CO_2-gassed) Krebs Henseleit solution at 37°C for the duration of the ischaemic period. This maintained temperature within the left ventricle at 36-37°C, as measured using a thermistor inserted via the left atrium. Following reperfusion with oxygenated perfusate, hearts were allowed to re-equilibrate for 10 minutes before injection of cumulative bolus doses of endothelins (10-70µl). A single dose response curve was produced in each preparation as endothelin responses were affected by previous exposure to the peptide. This protocol was repeated for comparison of all vasoactive agents used. Vehicle boluses were included in all experiments in the volume of the largest agonist injection and no injection artefact was noted.

A control group of hearts underwent perfusion alone for the total experimental time (110 minutes) and was compared with a group of hearts which underwent the ischaemia / reperfusion protocol, again without addition of agonists.

2.3 Effect of perfused indomethacin and L-NMMA on responses to endothelin-3

Following initial equilibration, a combination of indomethacin (10μM) and the L-arginine derivative N^G monomethyl L-arginine (L-NMMA, 100μM) was perfused, in order to inhibit both cyclooxygenase and nitric oxide synthase. Responses to a sub-maximal 50pmol dose of endothelin-3, and to 10nmol sodium nitroprusside, 1nmol bradykinin and 0.5u thrombin were compared following 15 minutes' equilibration with the inhibitors. Initial attempts to perform paired comparisons of responses before and after perfusion of the inhibitors were not continued as perfusion pressures were significantly higher at the time of the second agonist doses in both control and treated preparations. Hence separate preparations were used for treated and control (vehicle only) responses.

2.4 Studies using glibenclamide

After initial equilibration, hearts were perfused with either glibenclamide 10μM or vehicle (dimethyl sulphoxide). Following 15 minutes' equilibration with glibenclamide or vehicle, the response to a single bolus injection of the potassium channel opener lemakalim (10nmol) was assessed in order to demonstrate the effectiveness of the concentration of glibenclamide used. A single dose-response curve to endothelin-3 was then performed in each heart.

2.5 Studies using the perfused ET_A receptor antagonist BQ-123

Following equilibration, hearts were perfused with 1μM BQ-123 for 20 minutes before injection of bolus doses of endothelin-1 or endothelin-3 to produce cumulative dose-response curves. Preliminary studies were carried out similarly to test the selectivity of sarafotoxin 6c for ET_B receptors, but using a single sub-maximal dose of sarafotoxin 6c, (10pmol) following perfusion of 2μM BQ-123 for 10 minutes. The effect of this

concentration of BQ-123 at ET_A receptors was tested by subsequent injection of endothelin-1 (50pmol).

2.6 Desensitisation of ET_B receptors using sarafotoxin 6c

The rapid desensitisation of the ET_{B1} receptor-mediated vasodilator response (Le Monnier de Gouville & Cavero, 1991) was exploited by repeated administration of a maximal 100pmol dose of the selective ET_B receptor agonist sarafotoxin 6c.

2.6.1 Selection of interval between doses

The Krebs Henseleit perfusate used contained the lower potassium concentration indicated in section 2.1 (3.2mM) in order to raise coronary tone and thus reveal the full extent of sarafotoxin 6c-induced vasodilatation. Following equilibration, four 100pmol doses of sarafotoxin 6c were administered to each preparation with inter-dose intervals of 10, 20 and 30 minutes. The order of the dose intervals was randomised (using a Latin square) for each heart, to eliminate the possibility of influence from the number of previous doses. Results of this are seen in figure M-1, the 10 minute interval giving the greatest degree of desensitisation.

2.6.2 Selection of number of doses used

The lower potassium concentration indicated in section 2.1 was perfused as in the previous section. A sarafotoxin 6c dose was repeated at 10 minute intervals until desensitisation was apparent. After 3 doses, the dilatation to sarafotoxin 6c was reduced by over 90% as illustrated in figure M-2.

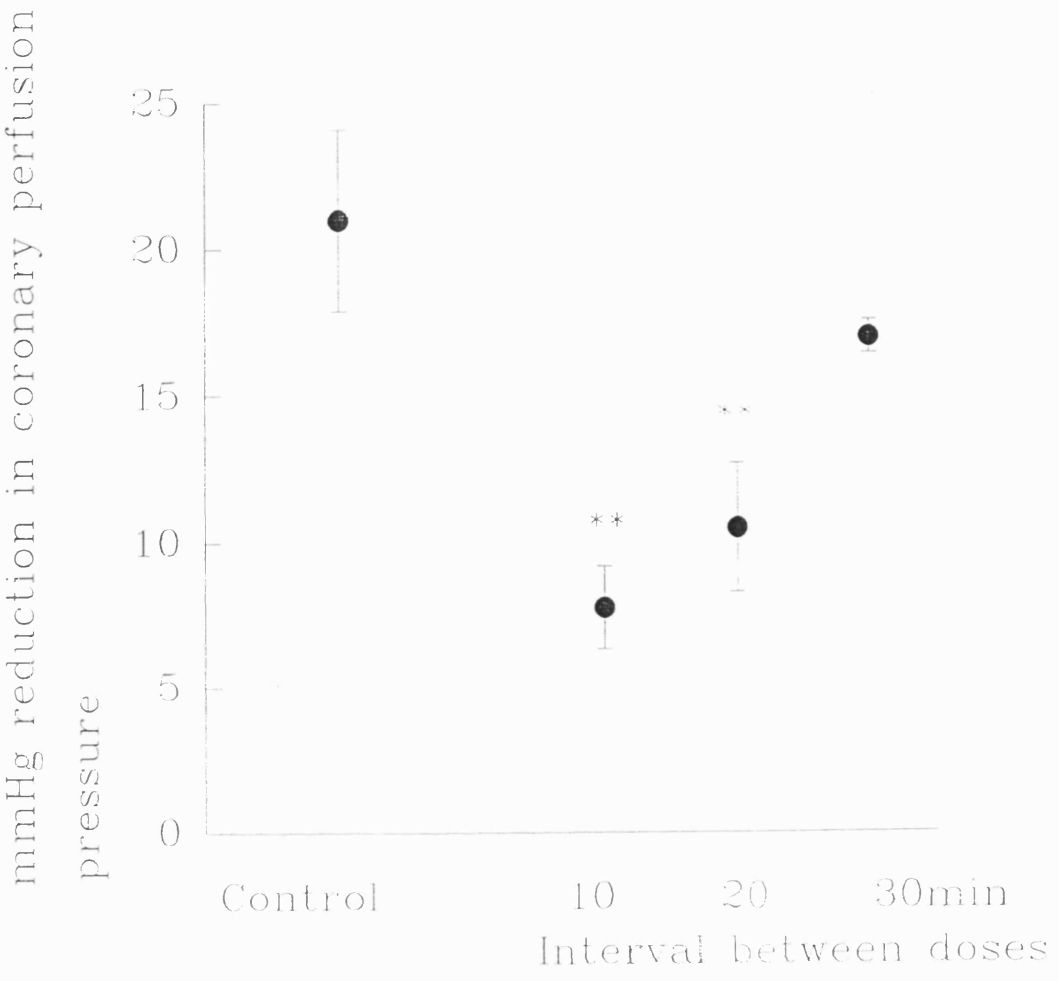


Figure M-1
The effect on the vasodilator response to a 100pmol dose of sarafotoxin 6c in the isolated rat heart preparation, of repeating the dose at a 10, 20 or 30 minute interval after the initial dose. Results are shown as mean ± SEM reduction from basal perfusion pressure (of 83 ± 3 mmHg) for n=4 preparations.
** denotes $P<0.01$ when compared with control vasodilatation (initial response) using two-way ANOVA and *post hoc* Tukey's test.

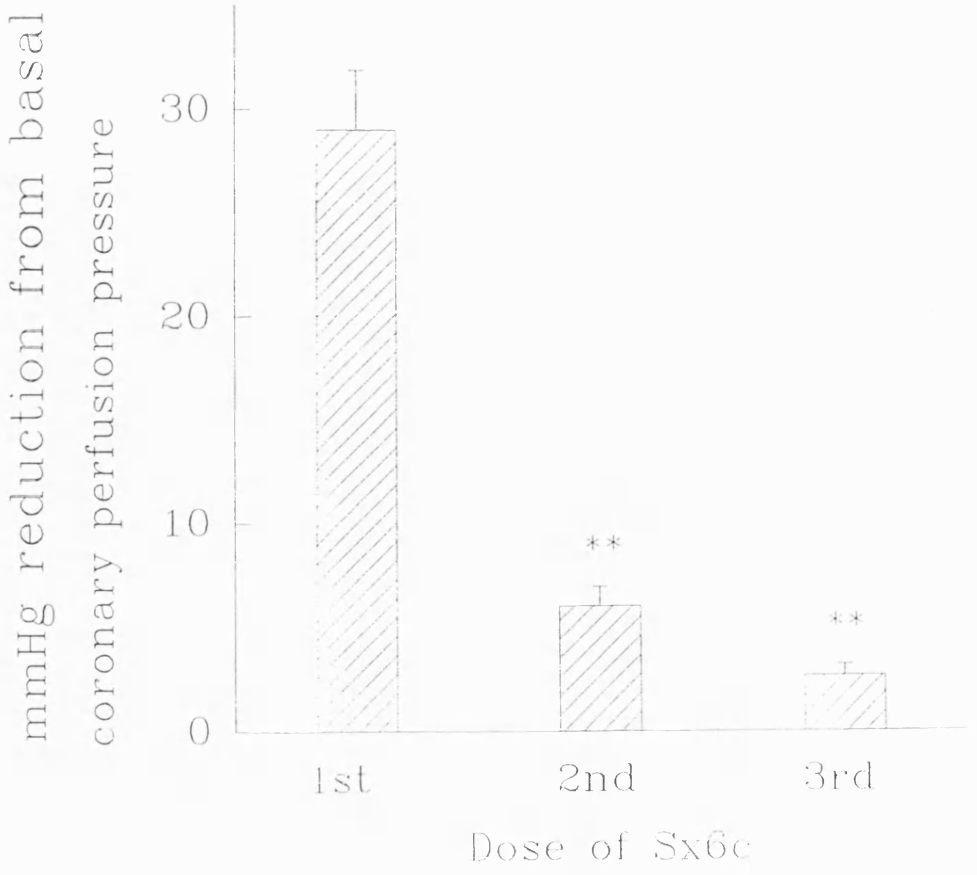


Figure M-2
The influence of the number of repetitions at 10 minute intervals of a bolus dose of sarafotoxin 6c, on the magnitude of the vasodilator effect in the isolated rat heart preparation. Effects of first, second and third successive doses shown as mean (± SEM, n=10) reduction from basal perfusion pressure (of 83 ± 3 mmHg).
** denotes $P<0.01$ when compared with the first dose by two-way ANOVA and *post hoc* Tukey's test.

2.6.3 Desensitisation of the vasodilator component of the endothelin-1 response

As a result of the information derived from experiments in sections 2.6.1 and 2.6.2 above, a protocol of three 100pmol doses of sarafotoxin 6c given at 10 minute intervals, was used. Hearts were perfused using modified Krebs Henseleit solution (see section 2.1) with the potassium concentration returned to the 5.9mM used in the ischaemia / reperfusion experiments. Sarafotoxin 6c desensitisation preceded the injection of a single sub-maximal (30pmol) dose of endothelin-1. In separate preparations, sub-maximal doses of the vasodilators bradykinin (10pmol) and sodium nitroprusside (10nmol) and the vasoconstrictors Bay K 8644 and phenylephrine were given following the same desensitisation protocol. Controls consisted of vehicle (saline) bolus injections in the same volume and at the same time points as the sarafotoxin 6c, prior to administration of vasoactive substances.

2.7 Materials used in perfusion experiments

Endothelins were obtained from Novabiochem U.K., Ltd. and sarafotoxin 6c from Peninsula Laboratories. These were dissolved in physiological saline solution to make a 10 or 100μM stock solution and further diluted in saline for bolus injection.

BQ-123 was a gift from Dr M. Sumner at Glaxo plc and a number of different stock concentrations were prepared in distilled water and stored at -20°C until required.

Bradykinin, sodium nitroprusside, adenosine, phenylephrine, thrombin, and papaverine were obtained from Sigma (Poole, Dorset); lemakalim was obtained from Beechams. All were prepared daily. Verapamil (Abbott Laboratories) was similarly prepared as required from the manufacturers' stock solution.

Bay K 8644 (Bayer AG) was dissolved in methanol to a stock concentration of 10mM, which was stored in an airtight container at -20°C until further dilution in physiological saline solution was required for use.

N^G monomethyl L-arginine, (Sigma) was initially dissolved in distilled water and sonicated prior to final dilution in Krebs Henseleit perfusate.

Indomethacin (Sigma, Poole, Dorset) was initially dissolved in a minimum volume of 1M Na_2CO_3 prior to final dilution in Krebs Henseleit solution.

Glibenclamide was dissolved in dimethylsulphoxide (Sigma, DMSO) to give a stock solution of 10mM prior to dilution in Krebs Henseleit perfusate. Vehicle control perfusate for glibenclamide consisted of a 1:1000 dilution of DMSO alone.

NaCl, KH_2PO_4, $CaCl_2$, $NaHCO_3$, D-Glucose, $MgSO_4$, KCl, $NaCO_3$ and other salts were obtained from BDH chemicals, Poole, Dorset.

Sagatal (sodium pentobarbitone) was obtained from RMB Animal Health Ltd, Dagenham.

3.0 Cell signalling

3.1. Culture methods for porcine coronary smooth muscle cells

3.1.1 Cell explantation

Materials and solutions (see section 3.3).

Buffer for collection of porcine hearts:

Sterile Hanks buffered salt solution (HBSS) with additions:

Gentamicin 200μg.ml^{-1} (recommended dose where contamination known to exist or is likely)

Streptomycin / penicillin mixture 100u.ml^{-1} each

Amphotericin B (Fungizone) 0.5μg.ml^{-1}

Growth medium:

Sterile Dulbecco's Modified Eagle's Medium (DMEM), with additions:

Streptomycin / penicillin mixture 100u.ml^{-1} each. Amphotericin B (Fungizone) 0.5μg.ml^{-1}

10% heat-inactivated foetal calf serum (FCS)

Methods

Hearts from adult pigs were collected from a local abattoir within 30 minutes of slaughter and the coronary arteries flushed via the aorta, using ice-cold HBSS. The hearts were transported and kept on ice until coronary excision.

A wedge of myocardium containing the right coronary artery was excised from each heart under aseptic conditions. This was done by making a first incision from the aortic root through the right ventricle to the posterior septum 1-2cm below the line of the right coronary artery. This was located by following the visible streak of periarterial fat. A second incision was then made in the right atrium 1cm above the line of the coronary artery.

The wedge was freed and kept in ice-cold HBSS in a sterile container until the following stages could be carried out.

The remainder of the explant procedure was performed in a laminar flow cabinet using a sterile technique and instruments. The coronary arteries were removed from the wedges and placed into growth medium until required. Each artery was then trimmed of excess fat and pinned out on a dissection board before being cut longitudinally, leaving the endothelial surface upward. The artery was kept moist at all times with DMEM. Endothelium was removed using a scalpel blade drawn over the inner surface and this procedure was repeated. The artery was then cut into small (2-5mm) squares which were then placed, tunica media side down, onto the growing surface of an upright T75 culture flask. A spatula was used to position the patches at 1cm intervals and leaving 2cm at the bottom of the flask for growth medium to be added. The patches were each moistened by addition of a minimum amount of DMEM by capillary attraction, and the bottom of the flask was covered with medium before placing in the incubator under an atmosphere of 5% CO_2: 95% air, at 37°C for 3 hours to allow adherence. After this time, the flasks were carefully laid down flat, allowing the DMEM to cover the tissue and were left undisturbed for 3-5 days to allow cells to explant. Cells could be seen growing out from the arterial patches at 5-10 days following dissection. Feeding was performed every 5 days by removal of old DMEM and any loosened arterial patches, and replacing it with fresh solution.

3.1.2 Cell passaging

When cells grew to a confluent monolayer, each T75 was passaged into 2xT150 flasks. This rapid expansion to a larger surface area for cell growth allowed rapid production of the cell populations necessary for experimental use whilst using cells at as low a passage number as possible (below passage 10), as some cell characteristics have been shown to change following repeated passaging (see discussion).

Materials and solutions (see section 3.3)

Trypsin / EDTA solution (1:250 ratio) at 37°C: (T/E, Trypsin 0.5g.l^{-1} with ethylenediaminetetraacetic acid, 0.2g.l^{-1}, in saline).

Phosphate-buffered saline without calcium and magnesium (PBS) at 37°C.

Method

Cells were washed twice using 10ml PBS at 37°C, and sufficient T/E mixture was added to cover the monolayer. Cells were incubated with the enzyme mixture for 2-5 minutes under frequent observation to minimise cell damage while ensuring maximum cell numbers were harvested from flasks.

When cells were fully suspended the T/E was diluted in excess DMEM containing 10% FCS which contains anti-trypsin.

The remaining enzyme mixture was removed by centrifugation at 1000rpm for 7 minutes and cells were resuspended in DMEM of the required volume for the size of flask used (e.g. 30ml for each T150 flask). Cells adhered to the growth surfaces of flasks after 2-4 hours and regained their typical flattened, elongated shape overnight.

3.1.3 Storage of cells in liquid nitrogen

To preserve excess cells at low passage number (see section 3.1.2) for future use, cells at 2nd or 3rd passage were frozen for storage in liquid nitrogen.

Materials and solutions (see section 3.3)

DMSO

FCS

DMEM, with added antibiotics as above (section 3.1.1)

Method

Washed, passaged cells from a confluent T150 flask resuspended to 1ml were divided into 4 aliquots of 0.25ml (approximately 8x10^5 cells each) before addition of the freezing mixture

of 0.2ml FCS and 0.05ml DMSO to give final concentrations as follows: cells 50%, FCS 40% and DMSO 10%. The cells were then frozen down in cryotubes by slowly lowering them into liquid nitrogen, immersing cells gradually over 6 hours using a commercially available cryotube holder with winding mechanism, and thus decreasing their temperature slowly. For use, cells were thawed rapidly at 37°C, rapidly mixed in 10ml of DMEM and washed in DMEM twice before final resuspension in medium and plating into flasks.

3.1.4 Identification of smooth muscle cell phenotype

Cells were observed under the light microscope and when confluent, the "hill and valley" appearance typical of vascular smooth muscle cells (VSMC) was as described by Chamley-Campbell *et al.*, (1979). See figure M-3.

For further confirmation, it was necessary to exclude the possibility of contamination by other cell types such as fibroblasts and endothelial cells. The immunocytochemistry staining method used was selective for a smooth muscle cell-specific actin as described by Skalli *et al.*, (1986) and this is outlined below. Antibody concentrations were optimised in preliminary experiments.

Materials and solutions (see section 3.3)

Dulbecco's phosphate-buffered saline (DPBS) containing both magnesium and calcium (each at 1mM)

Primary antibody: Monoclonal Anti-α_1 smooth muscle actin antibody (mouse IgG2a). 1:500 dilution in DPBS.

Secondary antibody: Rabbit anti-mouse IgG (Fab fragment), conjugated to Fluorescein (FITC): 1:200 dilution in DPBS.

Blocking serum : Normal rabbit serum. 1:200 dilution in DPBS.

Ice-cold methanol.

1:1 glycerol:DPBS mixture for fixing slides.

Methods

Cells grown to sub-confluence on glass coverslips were washed twice in DPBS to remove any remaining serum before being immersed in 100% methanol at -20°C for 10 minutes. It was found in preliminary experiments that if cells were fixed in this way, further permeabilisation was unnecessary. Following this, cells were again washed twice in DPBS and incubated in blocking serum for 1 hour at room temperature to block non-specific binding of the secondary antibody. Following repeated washes as previously, coverslips were incubated at room temperature in anti-α_1 smooth muscle actin primary antibody for 1 hour. Washes were repeated as described. Coverslips were again incubated at room temperature in the secondary rabbit anti-mouse antibody for 1 hour. Two final washes in DPBS were performed and coverslips were mounted on glass slides with a 1:1 glycerol:DPBS mounting solution. Slides were kept refrigerated in the dark until viewed under fluorescence microscopy using an excitation wavelength of 495nm (and emission at 525nm).

A known fibroblast cell preparation (human synovial fibroblast primary cell culture) was also grown on coverslips as a negative control, and specificity of the secondary antibody was demonstrated by omission of the primary antibody on some coverslips.

Positive staining was identified by eye under fluorescent light, and negative staining by microscopic comparison of cell numbers visible under tungsten light with numbers of cells having fluorescent filaments.

Photography was performed using a Nikon Optiphot-2 microscope with an Olympus OM2 camera. Optimum exposure times were determined in preliminary studies to be around 30 seconds using *Kodak ektachrome ELITE* 400/27° film.

Cell purity

Following application of the above staining technique, > 99% of all cells cultured from coronary arteries stained positive for α-smooth muscle actin. No synovial fibroblasts were identified which stained positively for α-smooth muscle actin, and where the primary

antibody was omitted, no fluorescence was detectable. The positively stained cells can be seen in figure M-4.

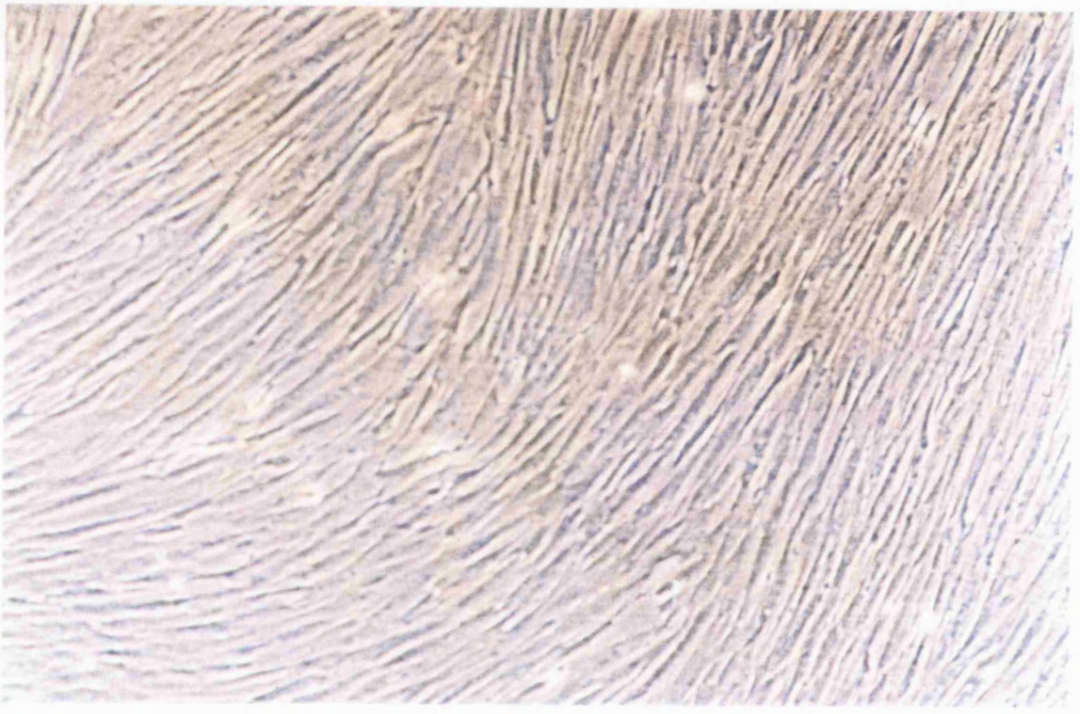


Figure M-3
Photomicrograph of a monolayer of porcine coronary smooth muscle cells grown to confluence and displaying typical "hills and valleys" characteristics. Taken under x100 magnification using Kodak ISO 64 tungsten film.

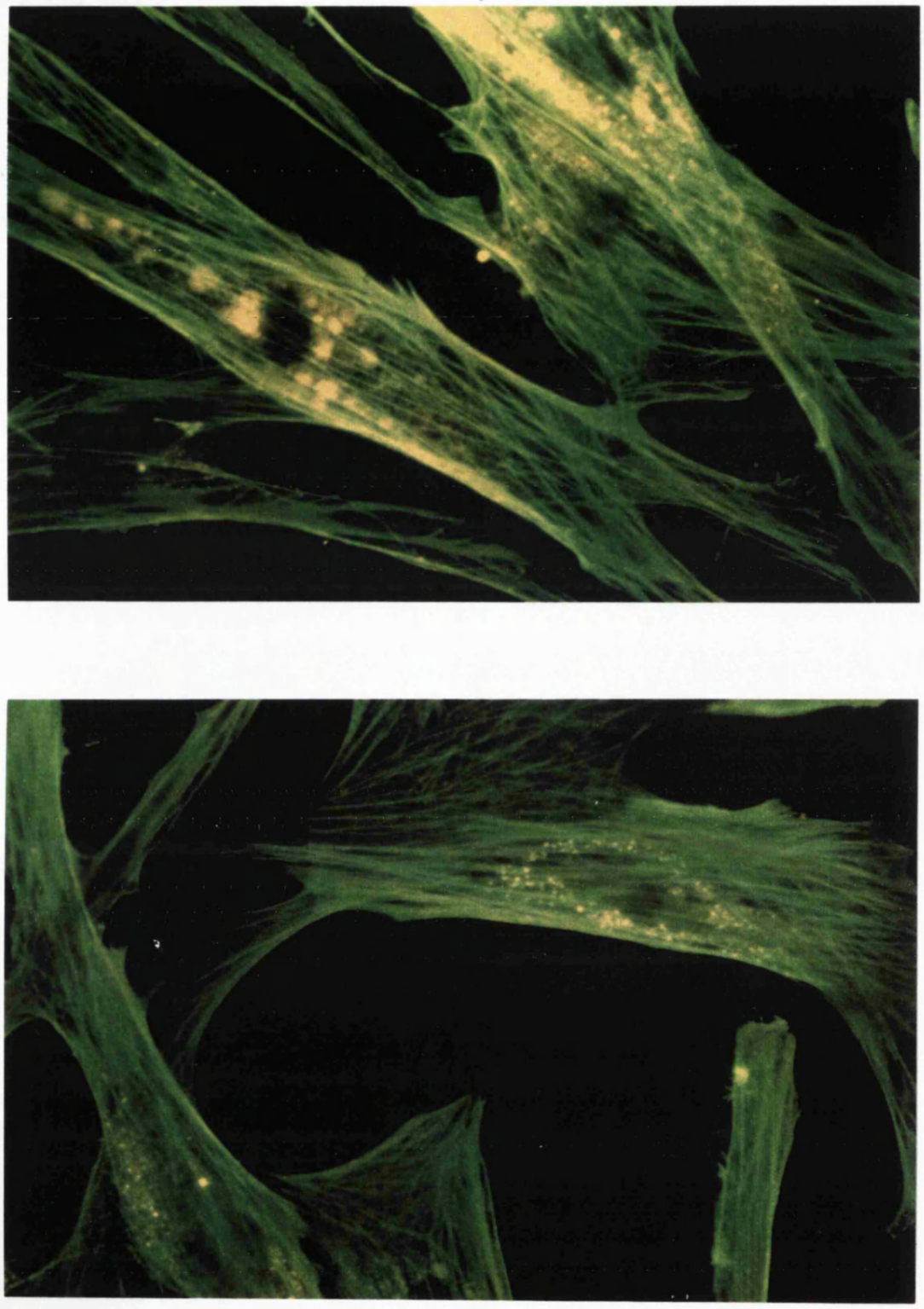


Figure M-4
Photomicrographs of examples of porcine coronary artery smooth muscle cells, following staining with anti α-smooth muscle actin primary antibody,and FITC-conjugated secondary antibody (see section 3.1.4). according to the method of Skalli *et al.*, (1986).

3.2 Calcium measurements

3.2.1 Cell culture techniques for calcium measurements in single cells, monolayers and cell suspensions

Cells at early passage numbers (2 to 6) were used as smooth muscle cells in culture may change phenotype following multiple passage (e.g. Eguchi *et al.*, 1994; Yamamoto *et al.*, 1983). Cells were grown in 96 well tissue culture plates (NUNC) and on glass coverslips (Chance-Propper #1) for measurement of calcium in single cells and groups of cells in monolayers. Cells were grown in T150 tissue culture flasks where large cell numbers were required for use in suspensions at a density of 10^6 cells.ml^{-1} (for total cell volume in each cuvette of 1.5ml). From preliminary assessments, it was determined that each T150 flask contained approximately 3-4x10^6 cells when grown to confluence, hence the number of flasks required for each experiment could be estimated. For experimental use, serum deprivation was required. This was firstly in order to promote cell differentiation, as cells in culture become "modulated" and undergo phenotypic change (Chamley-Campbell *et al.*, 1979). Secondly, it was noted in preliminary experiments, that addition of 10% FCS to cells during fluorimetry resulted in a large increase in intracellular calcium, hence exposure to high levels of growth factors and other constituents of serum was reduced prior to cell use. Serum deprivation was performed by leaving cells in 0.1% FCS in DMEM for a minimum of 24 hours, following the method of Little *et al.* (1992). After serum starvation, there was no apparent increase in cell numbers when counted under light microscopy.

3.2.1.1 Culture of cells in 96 well plates

Cells were passaged into the central 60 wells of a plate at an initial density of 10^5 cells per well, a seeding density which rapidly allowed near-confluent cell growth. Outer wells were filled with medium alone to increase humidity. Cells were cultured for up to 4 days in the plates before use and deprived of serum for 24-48 hours.

3.2.1.2 Culture of cells on glass coverslips

In initial experiments, cells were passaged into 6 well plates containing one 22mm diameter glass coverslip per well. The cells were left to attach in DMEM containing 10% FCS. However, cells were detaching from the glass after 24-48 hours, despite attempts to prolong this period using a variety of proteins (gelatin, collagen, laminin, and fibronectin) to coat the glass.

A marked loss of cells away from edges of multi-well plates was noted and to avoid this effect, sterile coverslips were inserted into T150 culture flasks via the flask neck. Using this technique, cells were adherent for at least 7-10 days. Hence this method was used to grow cells to confluence whilst allowing a consistent period of serum deprivation. This technique permitted the assessment of calcium responses in single cells grown as part of a monolayer or in groups of cells within the monolayer.

3.2.1.3 Culture of cells in T150 flasks

Cells were passaged into T150 flasks (Falcon) as described previously, and allowed to grow to full confluence prior to use for calcium measurements in cell suspensions. Where incubation with an antagonist was required for a longer period, this was added to the flask during serum deprivation.

3.2.2 Loading of cells with the calcium indicator fura-2

Materials and solutions (see section 3.3)

Assay buffer:

HEPES-buffered Tyrode (HBT) at room temperature. (HEPES buffering to prevent increase in pH associated with loss of dissolved CO_2 with bicarbonate buffering).

Composition (mM unless stated):	
NaCl	145
HEPES	10
NaH_2PO_4	0.5
KCl	5

D-glucose	5.55
Calcium	1
$MgCl_2$	1
Bovine serum albumin	0.25%

Fura-2/acetoxymethylester (AM) mixed with equal volume of 25% Pluronic F-127 prior to final dilution in HBT to 5μM

3.2.2.1 Loading of cells on 96 well plates

Cells were washed twice with HBT to remove any trace of remaining serum, which would contain esterases. Cells were then covered using the fura-2/AM / Pluronic F-127 mixture which was added to each well, and incubated for 30 minutes in the dark at 37°C. The fura-2/AM was pre-mixed with the non-ionic surfactant Pluronic F-127, a dispersing agent which prevents formation of precipitate of fura-2 and improves loading. Precipitation was visible as bright localisations of fluorescence under microscopy in preliminary experiments where Pluronic F-127 was not used. Following loading, the cells were washed twice to remove any extracellular fura-2/AM which is fluorescent but calcium insensitive and would interfere with recording of calcium responses. VSMC were then left to equilibrate in HBT containing 1mM calcium for 30 minutes, prior to use. This allowed intracellular de-esterification of fura-2/AM to produce the calcium-sensitive fura-2.

3.2.2.2 Coverslips

Coverslips were transferred to 6 well plates and washed twice in HBT before loading using a similar technique to that used for 96 well plates (section 3.2.1.1)

3.2.2.3 Cell suspensions

Harvest from flasks:

Dissociation of VSMC from flasks required a technique different from that used for passaging, as trypsin digestion would cleave extracellular domains of membrane proteins, including receptors. Hence calcium was chelated using EDTA to aid dissociation.

Additional materials and solutions (see section 3.3)

PBS (calcium and magnesium-free), at 37°C

EDTA dissociation buffer: in milli-Q de-ionised water and at 37°C

Composition (mM unless stated):

NaCl	130
KCl	6
NaH_2PO_4	1.2
HEPES buffer	20
EDTA	1
D-Glucose	11.5
BSA	0.1%

pH to 7.4 using 2M NaOH solution

HEPES-buffered DMEM:

DMEM to which was added 26mM HEPES

pH checked and adjusted to 7.4 if necessary using 2M NaOH or HCl

Methods

Cells were washed twice using calcium / magnesium-free PBS to remove DMEM (which contains calcium and magnesium). ETDA buffer (5ml to cover the cells) was added to each flask, tapping to assist recovery until cells were dispersed (approximately 20 minutes). After the VSMC were dissociated, 10ml of HBT containing 1mM calcium was added to each flask to replace chelated calcium, and this was repeated to wash the maximum number of cells from each flask. Cells were then centrifuged at 1000rpm for 7 minutes. Cells were

resuspended thoroughly by trituration and diluted to a volume of 20ml HBT to wash off any remaining EDTA before being re-centrifuged.

Loading

All steps using fura-2/AM were performed with light excluded to reduce photo-bleaching of the dye.

The cell pellet was resuspended in 5ml HEPES-buffered DMEM to which had been added the fura-2/AM / Pluronic F-127 mixture. The cells were stirred gently during loading to prevent aggregation and to allow even distribution of the fura-2/AM. This was performed using a magnetic stirrer on low setting, for 45 minutes at room temperature. Cells were then washed twice by centrifugation and resuspension in 30ml volumes of HEPES-buffered DMEM to remove any remaining extracellular fura-2/AM. Finally, cells were resuspended to a concentration of $1x10^6$ cells per ml in DMEM and divided into 1.5ml aliquots. The cells were equilibrated at room temperature for 30 minutes to allow maximal intracellular de-esterification of the fura-2/AM as described above. Immediately prior to use, VSMC were briefly spun using an Eppendorff centrifuge, and resuspended in 1.5ml warmed HBT assay buffer before addition to the cuvette.

3.2.3 The fluorimetry set-up

The calcium signal was measured using a Photon Technology International (PTI) Deltascan fluorimeter with a fibre optic light-guide. This could be used to illuminate a microscope stage or a cuvette system as required. Excitation wavelengths for fura-2 were set at 340 and 380nm using monochromators, and these wavelengths were alternated at a rate of 60Hz via a rotating mirror (chopper). Emitted light at 510nm was detected using a photomultiplier tube and the image differentiated using PTI software into 340/380 ratio. The microscopy setup required a dichroic mirror to allow passage to the stage of the excitation signal as well as to filter the 510nm emission wavelength in the opposite direction. Other refinements for the microscopy setup included a shutter which could be used to select an area under the microscope stage for fluorescence measurement.

3.2.3.1 96 well plates

A Nikon Diaphot inverted microscope was used to view 20-30 loaded cells and VSMC were viewed using a 20x fluorescence objective at room temperature.(the greatest magnification available with the focal distance required for the plate). The shutter was used to select an area containing ~20 VSMC in which calcium was to be measured.

3.2.3.2 The Intracell chamber for coverslips

Coverslips were secured in an Intracell coverslip holder and sealed using silicone rubber. The holder was then placed in a heating chamber (Intracell, Cambridge) and allowed to equilibrate to 37°C. After this period, single cells or groups of 5-10 cells were selected for measurement using the diaphragm. It was also possible to view single cells as the cells were viewed using a 40x 1.3F oil immersion quartz fluorescence objective. Use of this objective was possible due to the single layer of thin glass separating the cells from the lens.

3.2.3.3 The cuvette system

The cell suspensions were added to a cuvette and placed in a heated chamber warmed to 37°C. The fibre optic light guide was diverted from the microscope system to illuminate the cells and the detector wavelength was selected using the PTI software. A dichroic mirror was unnecessary as the excitation and emission wavelengths followed separate pathways.

3.2.4 Protocols for measurement of intracellular calcium in single cells and monolayers

3.2.4.1 Cells in 96 well plates

VSMC were left bathed in a known (250µl) volume of HBT to equilibrate and a field of view was chosen as described in section 3.2.3.1.

Basal calcium was measured, endothelin-1 added to the well (as a 1:10 dilution following removal of an equal 25µl volume) and subsequent change in intracellular calcium ($[Ca^{2+}]_i$) monitored. A single concentration of any one agonist was added to each group of cells as the initial added concentration remained in contact with the cells throughout and

desensitisation was possible. Second agonist additions were performed in the presence of the initial agonist but maintaining the same bathing volume by removal of 25µl before addition. For example where sarafotoxin 6c was used, the response to endothelin-1 was assessed afterwards, and a standard 1µM bradykinin concentration was added to wells after the addition of endothelin-1 in order to confirm cell viability where responses to the first agonist were lost or reduced.

In experiments where concentrations of BQ-123 were used, the antagonist was added to the cells at least 3 minutes prior to the addition of endothelin-1, again isovolumetrically. In a separate series of experiments, BQ-123 was added as above prior to the addition of bradykinin.

Where 5mM $NiCl_2$ was used to determine the effect of extracellular calcium on the endothelin-1 response, this was added immediately prior to the addition of endothelin-1, again keeping the bathing volume constant.

3.2.4.2 Cells on glass coverslips

VSMC were left bathed in 500µl final volume of HBT and allowed to equilibrate to 37°C for 60 seconds. Agonist addition (50µl volume) was performed as described in 3.2.4.1 and responses monitored for up to 6 minutes. The addition of BQ-123 was performed in a similar manner to that used in monolayers.

3.2.4.3 Cell suspensions

VSMC suspensions were added to the cuvette, maintained in suspension with a magnetic stirrer and allowed to equilibrate at 37°C for 1 minute. Recording of 340 and 380 signals was performed for a further minute prior to addition of a concentration of endothelin-1 in a 1:100 dilution. Separate cell suspensions were used for each endothelin concentration in the range of 1 to 300nM, again to avoid desensitisation (see section 3.2.4.1). Dose response curves were produced in the presence or absence of concentrations of the ET_A receptor antagonist BQ-123. The antagonist was added at either 3 minutes prior to the addition of endothelin-1 or simultaneously with the agonist. Where the tyrosine kinase

inhibitor herbimycin-A was used, it was added to culture flasks 20-24 hours prior to the experiment and these cultures were prepared separately from the controls.

3.2.5 Calibration of intracellular calcium concentrations from fluorescence ratios

3.2.5.1 Single cells and monolayers

The method of Grynkiewicz *et al.* (1985) was not suitable for cells under microscopy, as the maximum calcium signal could not be determined by cell lysis, the loss of fura-2 from the focused field of view being the result (Roe *et al.*, 1990). Measurement of the maximum calcium value using the non-fluorescent calcium ionophore, ionomycin was also unsuccessful as calcium did not equilibrate across the cell membrane, again a well recognised problem (ibid). "Maxima" thus obtained were consistently smaller than changes in $[Ca^{2+}]_i$ obtained using the agonist endothelin-1. Minima also were problematic as alkalinisation could result in cell lysis and again there were equilibration difficulties with EDTA. This method was abandoned, in favour of a series of calcium standard solutions which were added to successive wells or coverslips and resulting calcium readings in 1μM fura-2 free acid were fed into a look-up table on the PTI software.

3.2.5.2 Cell suspensions

As some flasks were prepared separately and cells were likely to form aggregates in suspension, calibration of the calcium signal was performed in each cuvette. This was done according to the method of Grynkiewicz *et al.* (1985). In brief, the maximum fluorescence signal was obtained by lysis of the cells in 1mM calcium using digitonin ($4mg.ml^{-1}$) 120μl per cuvette, and the minimum value found by chelation of calcium using 10μl of 1M EDTA, all performed following alkalinisation with 10μl of 2M NaOH solution. The equation used by Grynkiewicz *et al.* is as follows:

$$[Ca^{2+}] = Kd \frac{R - Rmin \times Sf2}{Rmax - R \times Sb2}$$

where K_d is the dissociation constant for the Ca^{2+} / fura-2 complex at 37°C (taken as 224nM and assuming constant intracellular ionic concentrations), R_{min} and R_{max} are the 340/380 ratios obtained in calcium-free and calcium-saturated conditions respectively, S_{f2} and S_{b2} are the fluorescence measurements at 380-nm in calcium-free and calcium-saturated conditions respectively and R is the 340/380 ratio from which the calcium concentration is to be derived.

3.3 Additional materials for cell culture and fluorimetry

Cell culture media and antibiotics were obtained from GIBCO, concentrated solutions being reconstituted and pH balanced where necessary, according to the manufacturers' recommendations.

Phosphate-buffered saline (PBS: Composition (mM) KH_2PO_4 1.5; NaCl 154; Na_2HPO_4 2.35) and Dulbecco's phosphate-buffered saline (DPBS: Composition (mM) $CaCl_2$, 1; KCl, 2.7; $MgCl_2$, 0.5; NaCl, 137; Na_2HPO_4, 8. pH balanced using 5N NaOH solution) were both obtained as 10x stock solutions from GIBCO and also prepared using deionised sterile water.

Other sterile plastics and tissue culture materials were all obtained from GIBCO except for T150 tissue culture flasks which were bought from Falcon.

Antibodies were obtained from Sigma Immunochemicals, and aliquoted before being stored at -20°C. Solutions in current use were stored at 4°C.

Fura-2/AM and fura-2 free acid (Molecular Probes Inc, USA). were dissolved in dry DMSO (Sigma) and stored at -20°C. Pluronic F-127 (Molecular Probes) was also prepared in dry DMSO but stored at room temperature for up to one week, being prepared freshly if any precipitate was seen. Ionomycin (Molecular Probes) was also dissolved in dry DMSO and kept refrigerated until required.

The standard calcium solutions were purchased as a kit from Molecular Probes. These were prepared by addition of fura-2 free acid in the minimum dilution volume to produce a final fura-2 concentration of 1μM.

Herbimycin-A was bought from GIBCO and dissolved in DMSO prior to storage at -20°C. Digitonin (BDH chemicals, Poole Dorset) was prepared as a 4mg.ml.$^{-1}$ stock solution as required.

Other chemicals used in preparation for and during the fluorimetry experiments were obtained from Sigma.

All agonists for fluorimetry were dissolved in calcium-free physiological saline solution

4.0 Statistical analyses

The statistical tests outlined below were applied to experimental data either by hand, or by application of the University of Bath *INSTAT* computer programme.

4.1 Acceptance of the null hypothesis

In the comparison of samples, the null hypothesis, i.e. the probability of the difference between them being obtained by chance alone (denoted "P"), was accepted at values of greater than $P>0.05$. The null hypothesis was rejected where $P<0.05$. Two-tailed tests were used in all cases.

4.2 Tests of the null hypotheses

4.2.1 Parametric tests

4.2.1.1 Student's t-test

This test was used to compare means of data from samples of control versus treated groups where only two means were to be compared. This test is more powerful that the equivalent non-parametric test (Mann-Whitney U-test), and is less likely to result in a type II error where the null hypothesis is erroneously accepted; hence small but significant differences between data groups are more likely to be revealed. Where appropriate, the paired t-test was used, comparing mean difference between pre- and post-test data in the same tissue preparation or sample. The criteria for selection of Student's t-test also included determination of equality of variances of the samples by use of Fishers F-test.

4.2.1.2 Analysis of variance

Where more than two means were to be compared, the above test was selected. Where a difference was identified between the means tested, the data groups were identified using an appropriate *post hoc* test as indicated.

4.2.2 Non-parametric tests

These tests were used where the variances of the samples used were unequal or otherwise did not fulfil the criteria for use of a parametric test. In a series of experiments where one group of the data required a non-parametric test the others in the set were also tested using a non-parametric test for continuity. The increased likelihood of a Type II error was considered preferable to the possibility of a Type I error where the null hypothesis would be erroneously rejected.

4.2.2.1 Mann Whitney U-test

This test is based on the summed ranks assigned to the data from both control and test preparations. Where means could not be compared using Student's t-test, this was the test of choice.

4.2.2.2 Wilcoxon's signed rank test

This test was used for data not fulfilling the criteria for parametric testing where control and experimental recordings were obtained from the same preparation, and is the equivalent of the paired t-test.

The statistical test used is indicated as appropriate in the results section.

RESULTS

5.0 Coronary reactivity

5.1 Effects of ischaemia and reperfusion alone

5.1.1 Effect of ischaemia followed by reperfusion on coronary perfusion pressure

Hearts which were simply perfused for a total 2 hour period (30 minutes' equilibration, and 90 minutes' perfusion) were compared with hearts which were subjected to 30 minutes of ischaemia after 30 minutes' equilibration and reperfused for 60 minutes. The last 50 minutes corresponded with the period of agonist administration in control and ischaemic / reperfused hearts.

In control hearts, during the last 50 minutes of perfusion, there was a steady rise in perfusion pressure, from a mean level of 96 ± 17 mmHg rising to a final mean recording of 111 ± 21 mmHg (mean ± sem, n=5). The corresponding period in ischaemic / reperfused hearts showed a starting mean perfusion pressure of 88 ± 7 mmHg rising to a final 116 ± 18 mmHg (n=4; figure R-1). There was no statistically significant difference between the control and the ischaemic / reperfused hearts when compared using Student's t-test, although there is a trend for a greater rate of rise in the ischaemic / reperfused hearts.

5.1.2 Effects of ischaemia and reperfusion on developed tension and heart rate.

The mean heart rate following equilibration was 318 ± 26 beats per minute (n=5) and this did not change significantly after 110 minutes' perfusion ($P>0.05$, n=5). Developed tension in control hearts was initially stable after equilibration at a mean level of 7.6 ± 1.0g (n=5) after adjustment to 2g resting tension. This showed a tendency to decrease with time, although the change from the initial recording was not statistically significant ($P>0.05$, n=5).

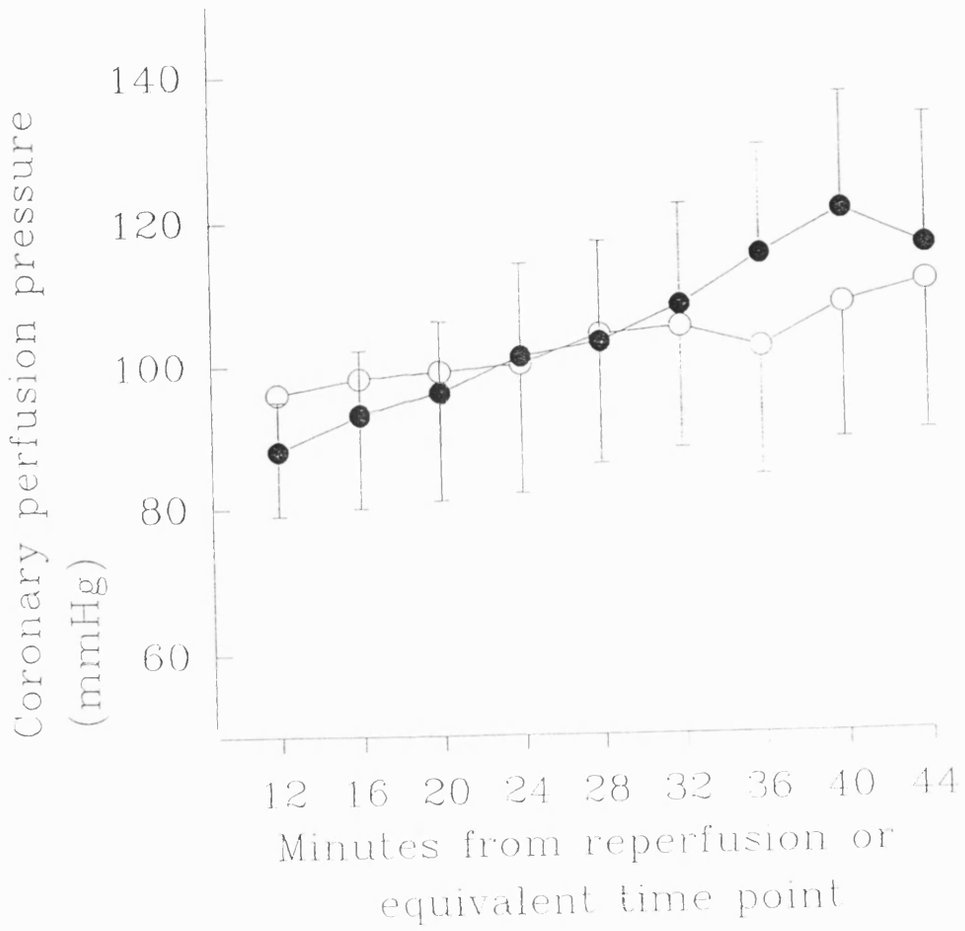


Figure R-1
The effect of time alone on coronary perfusion pressure in control (o-o) and ischaemic / reperfused hearts (●-●). Perfusion pressure is given as mean ± SEM for n=4 hearts. The period shown corresponds with that used for agonist administration in subsequent experiments. There are no significant differences between mean perfusion pressures at any of the individual time points (Student's t-test).

Ischaemia resulted in a loss of developed tension, and in the development of an ischaemic contracture, manifest as an increase in resting tension. The maximum increase in resting tension of 4.9 ± 0.6g (n=4) was recorded after a mean of 22 ± 1 minutes (n=4) of stopped flow. Following reperfusion, resting tension returned to near pre-ischaemia levels although recovery of developed tension was minimal (<2g) and was therefore not measurable with accuracy. The heart rate, triggered by the tension transducer was not therefore recordable after reperfusion.

Examples of traces showing the protocol, and responses to endothelin-1 in control and in ischaemic / reperfused hearts are seen in figure R-2a

5.2 Effect of ischaemia and reperfusion on coronary responses to endothelins and sarafotoxin 6c

5.2.1 Morphology of endothelin and sarafotoxin 6c responses in controls hearts

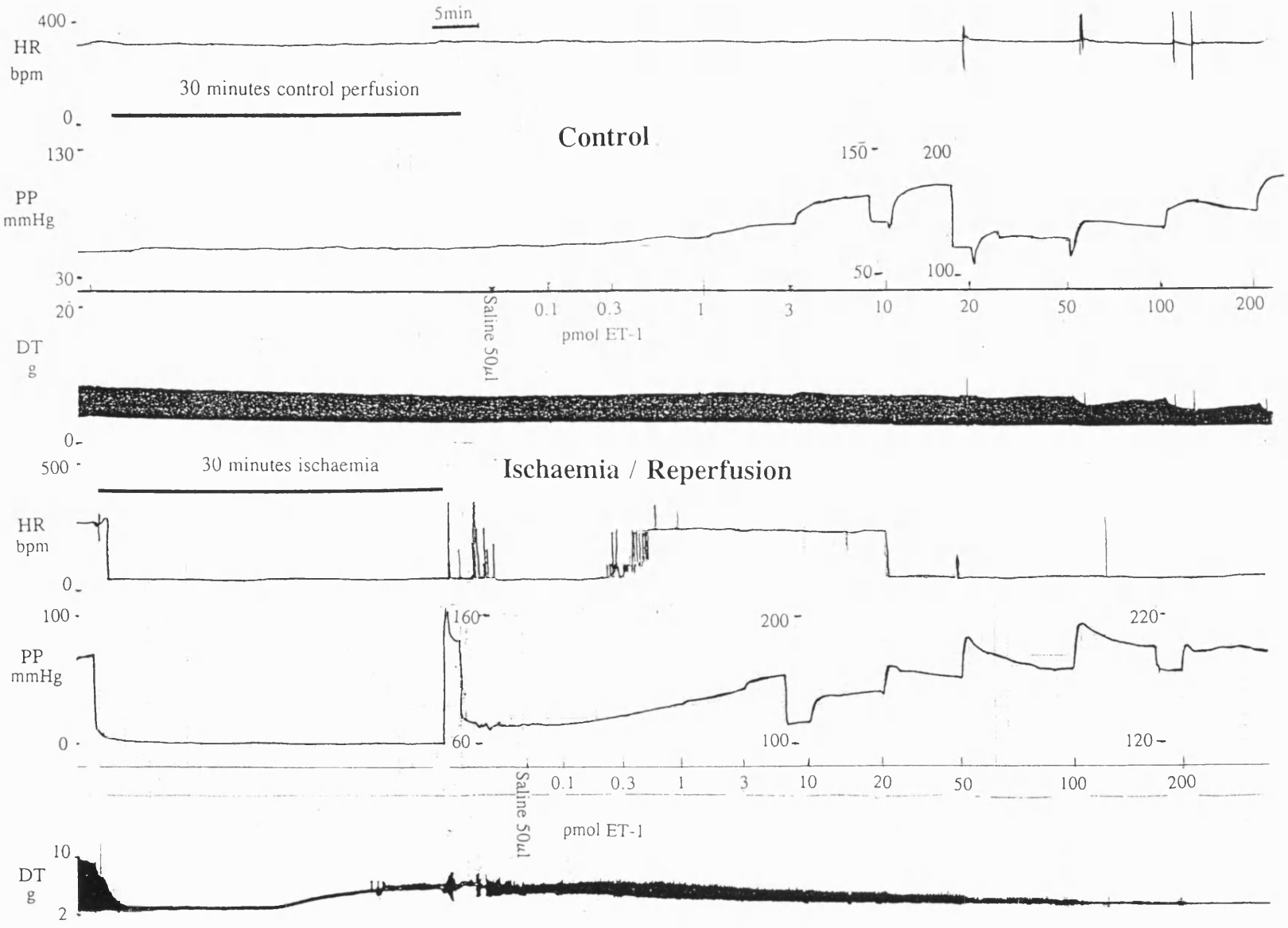
Endothelin-1 and endothelin-2 had dilator and constrictor actions in the coronary circulation (figure R-2). In the dose range producing coronary dilatation, a transient vasodilator phase was followed by a sustained vasoconstriction (plateau phase). At doses lower than the threshold for vasodilatation (e.g. 1-10pmol), the vasoconstrictor phase was slower in onset, and sustained. At doses of 200pmol and above, the vasodilator phase was absent, probably due to desensitisation (see section 5.7) and the onset of vasoconstriction was sharper.

Endothelin-3 was less potent as a vasoconstrictor than endothelin-1 and endothelin-2, the threshold for this phase of the response being ~200pmol. A vasodilator response was produced in a similar dose range to that of endothelins -1 and -2 (10-100pmol). The morphology of the vasoconstrictor responses was therefore different, vasodilatation occurring alone at the lower doses (up to 100pmol), and at higher doses (200pmol and above) the vasoconstrictor phase consisted of an initial peak preceding a return to near basal values, the plateau phase being comparatively small (figure R-3).

Sarafotoxin 6c produced a marked coronary dilator response at doses of 1-200pmol, but no vasoconstriction was apparent. The administration of a single 1nmol dose in separate

Figure R-2
Representative responses to three bolus doses of endothelin-1 (3, 20 and 200pmol) in normally perfused rat hearts. Upper traces show changes in perfusion pressure (PP) and lower traces developed tension (DT). Endothelin-2 responses were similar in morphology in a similar dose range to those to endothelin-1.

Figure R-2a
Two original traces representing the ischaemia reperfusion protocol followed in experiments described on page 52. The upper trace shows heart rate (HR), perfusion pressure (PP) and developed tension (DT) in a control preparation perfused for a period corresponding to the 30 minute period in the lower trace during which the heart was subjected to global zero flow ischaemia and superfused. Differences in the morphology of the responses are apparent after the ischaemic period in the lower trace when compared with the control.


400
HR
bpm
5min
30 minutes control perfusion
0
Control
130
150
200
PP
mmHg
30
50
100
20
Saline 50µl
0.1
0.3
1
3
10
20
50
100
200
pmol ET-1
DT
g
0
500
30 minutes ischaemia
Ischaemia / Reperfusion
HR
bpm
0
100
160
200
220
PP
mmHg
0
60
100
120
Saline 50µl
0.1
0.3
1
3
10
20
50
100
200
pmol ET-1
10
DT
g
2

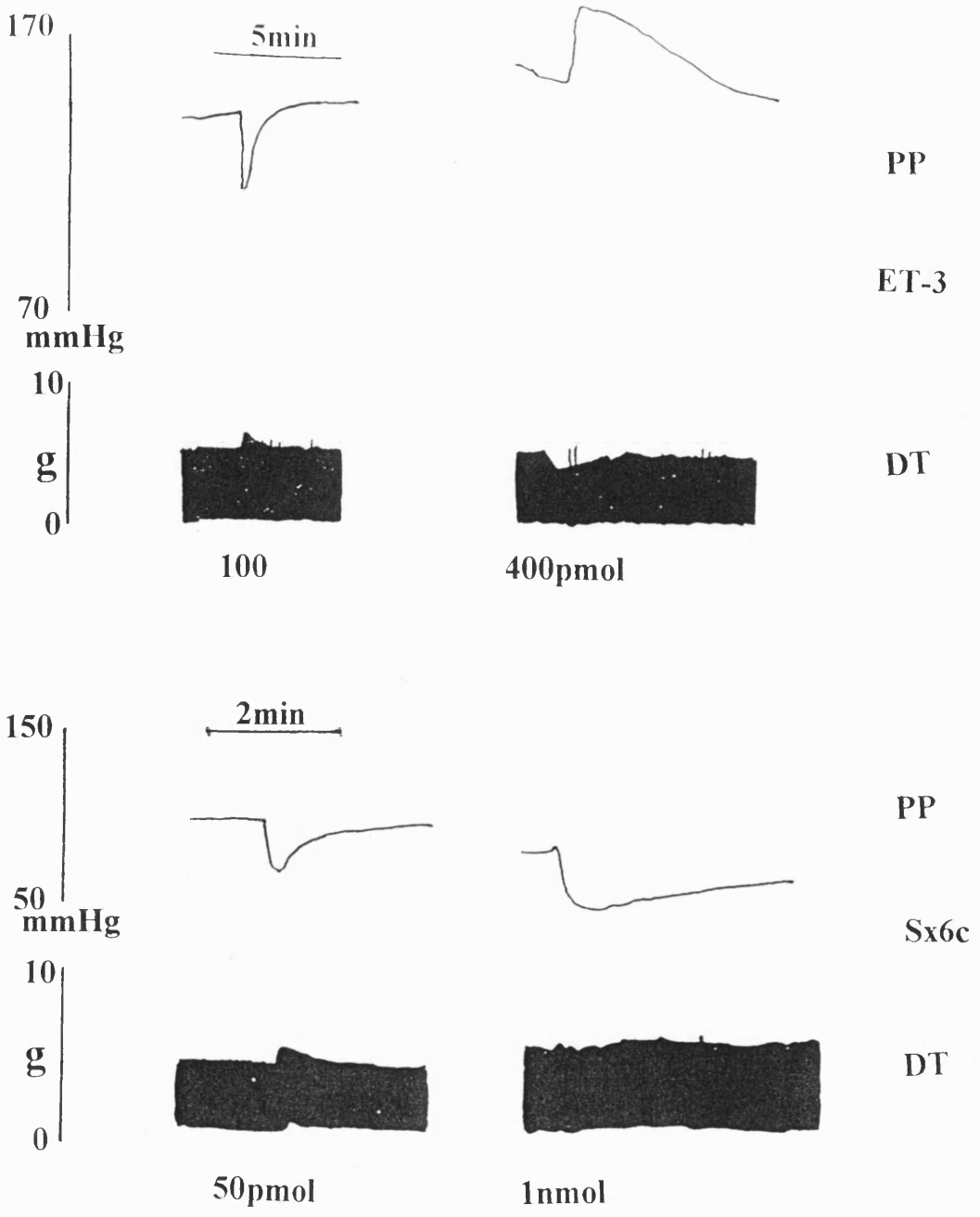


Figure R-3
The responses to two bolus doses of endothelin-3 (ET-3; 100 & 400pmol, upper traces) and sarafotoxin 6c (Sx6c; 50pmol & 1nmol, lower traces) in isolated perfused rat hearts, showing perfusion pressure changes (PP) and developed tension (DT). The ET-3 responses are from the same preparation, and were part of a series of increasing doses. Sx6c responses are shown to a 50pmol dose from one heart, and a 1nmol dose which was the sole dose administered in a separate preparation.

hearts produced vasodilatation but no accompanying vasoconstrictor effect, suggesting that the lack of vasoconstriction was not due to a desensitisation of the receptor involved (figure R-3). The vasodilator effect of a single higher dose of sarafotoxin 6c indicates that the diminishing effect after repeated bolus doses in one preparation (figure R-6) may be due to a desensitisation of this component of the response.

None of the endothelins nor sarafotoxin 6c had any significant effect on heart rate in control hearts ($P>0.05$). Developed tension was not significantly altered by endothelin-3 and sarafotoxin 6c, there being no significant difference between pre- and post-dose tension measurements over the full range of doses tested for each peptide. Higher doses of endothelins -1 and -2 suppressed developed tension as compared with pre-dose levels, this achieving statistical significance ($P<0.05$, Student's t-test) at the 200pmol dose where also the vasoconstriction was maximal.

5.2.2 Morphology of endothelin and sarafotoxin 6c responses in ischaemic / reperfused hearts

Following ischaemia and reperfusion, the vasodilator phases of the responses to endothelins and sarafotoxin 6c were severely compromised. This was accompanied by changes in the morphology of the vasoconstrictor phases of the responses which were sharper in onset for endothelins -1 and -2 (see figure R-4). This was less marked in the response to endothelin-3 where the onset of vasoconstriction was already more acute than in the response to endothelins -1 and -2. These changes in morphology are consistent with the loss of the vasodilator phase. Even with vasodilatation attenuated, sarafotoxin 6c did not produce any notable vasoconstrictor activity in ischaemic and reperfused hearts (figure R-4).

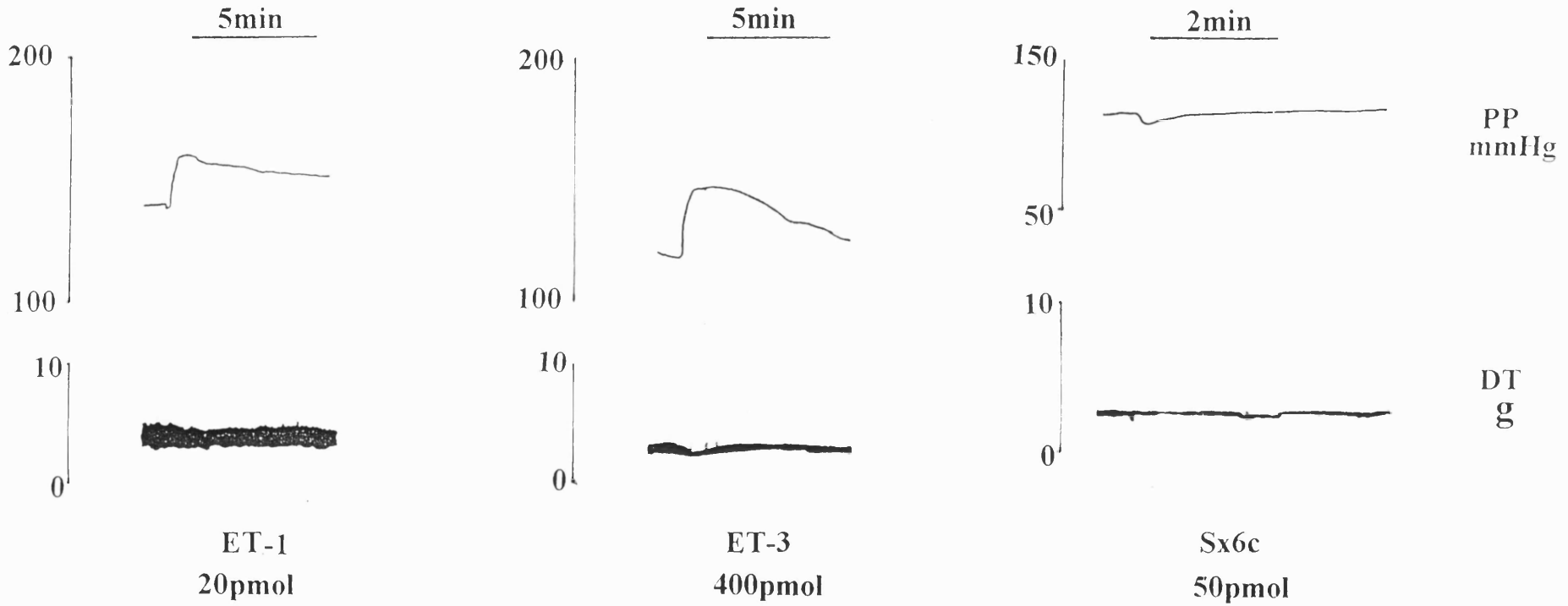


Figure R-4

The changes in perfusion pressure (PP, upper traces) in response to endothelins -1 (ET-1) and -3 (ET-3) and sarafotoxin 6c (Sx6c) in ischaemic / reperfused hearts, showing differences in morphology of the responses when compared with similar doses in figures R-2 and R-3. Lower traces show developed tension (DT), which is severely attenuated after ischaemia and reperfusion.

5.2.3 Effects of ischaemia / reperfusion on the magnitude of responses to endothelins and sarafotoxin 6c

5.2.3.1 Vasodilatation

As stated in the previous section, responses to endothelins and sarafotoxin 6c were altered by an attenuation of the vasodilator phase following ischaemia and reperfusion, and the difference from control responses achieved statistical significance ($P<0.05$) where the vasodilatation was marked, i.e. for endothelin-3 and sarafotoxin 6c. This is summarised in figures R-5 & R-6.

5.2.3.2 Vasoconstriction

The vasoconstrictor effect of all three endothelins was enhanced following ischaemia and reperfusion (figures R-7 & R-8a). although the difference between control and ischaemic / reperfused hearts was not statistically significant for all doses. It should be noted that the cumulative increases in perfusion pressure include any change in underlying perfusion pressure which might occur as a result of ischaemia alone (see figure R-1) The minimal vasoconstrictor responses to sarafotoxin 6c showed no enhancement following ischaemia and reperfusion (figure R-8b).

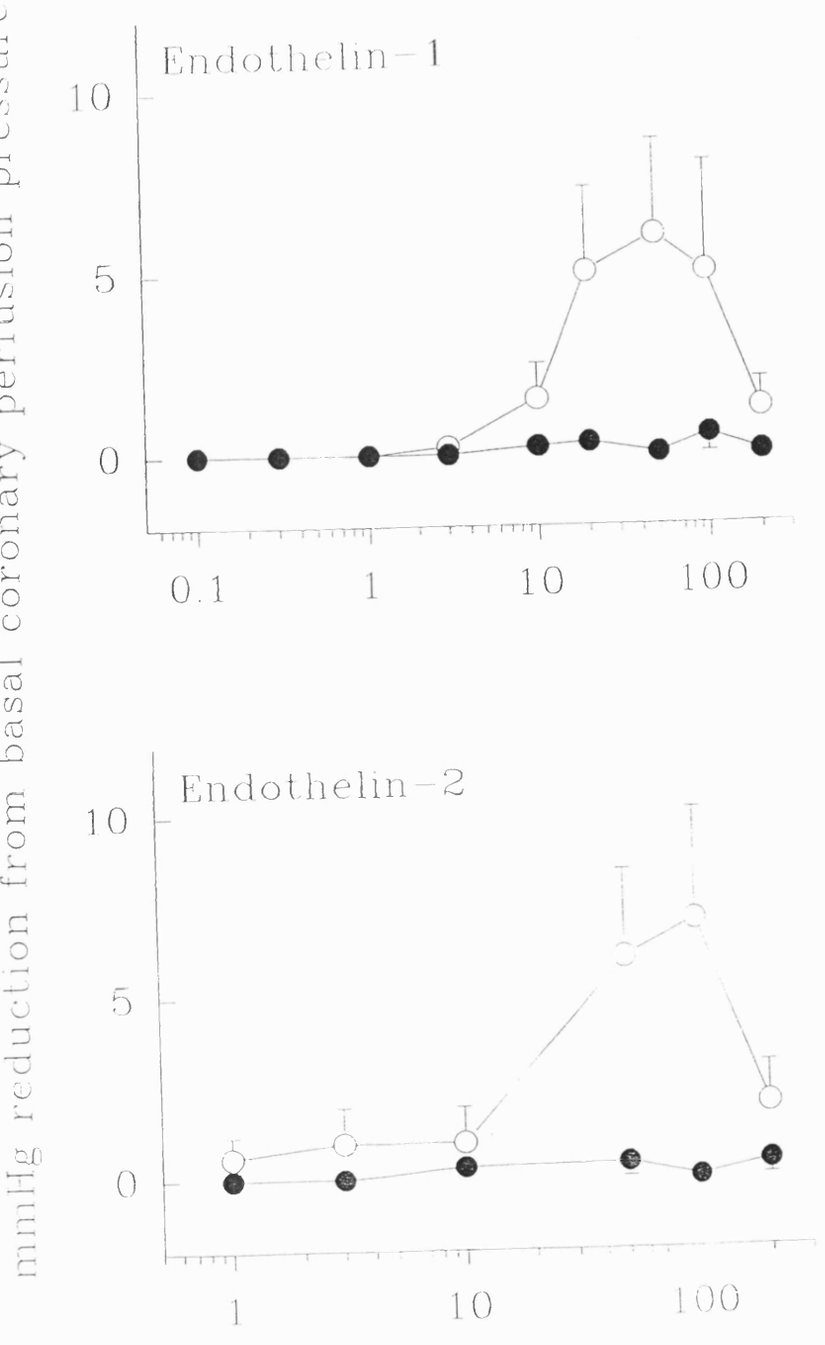


Figure R-5
The initial coronary dilator component of the responses to endothelin-1 and endothelin-2 (pmol) in control (o-o) and ischaemic / reperfused (●-●) hearts. Values are mean vasodilatation ± SEM (n=5-6). There are no statistically significant differences between the samples after analysis by Mann Whitney U-test.

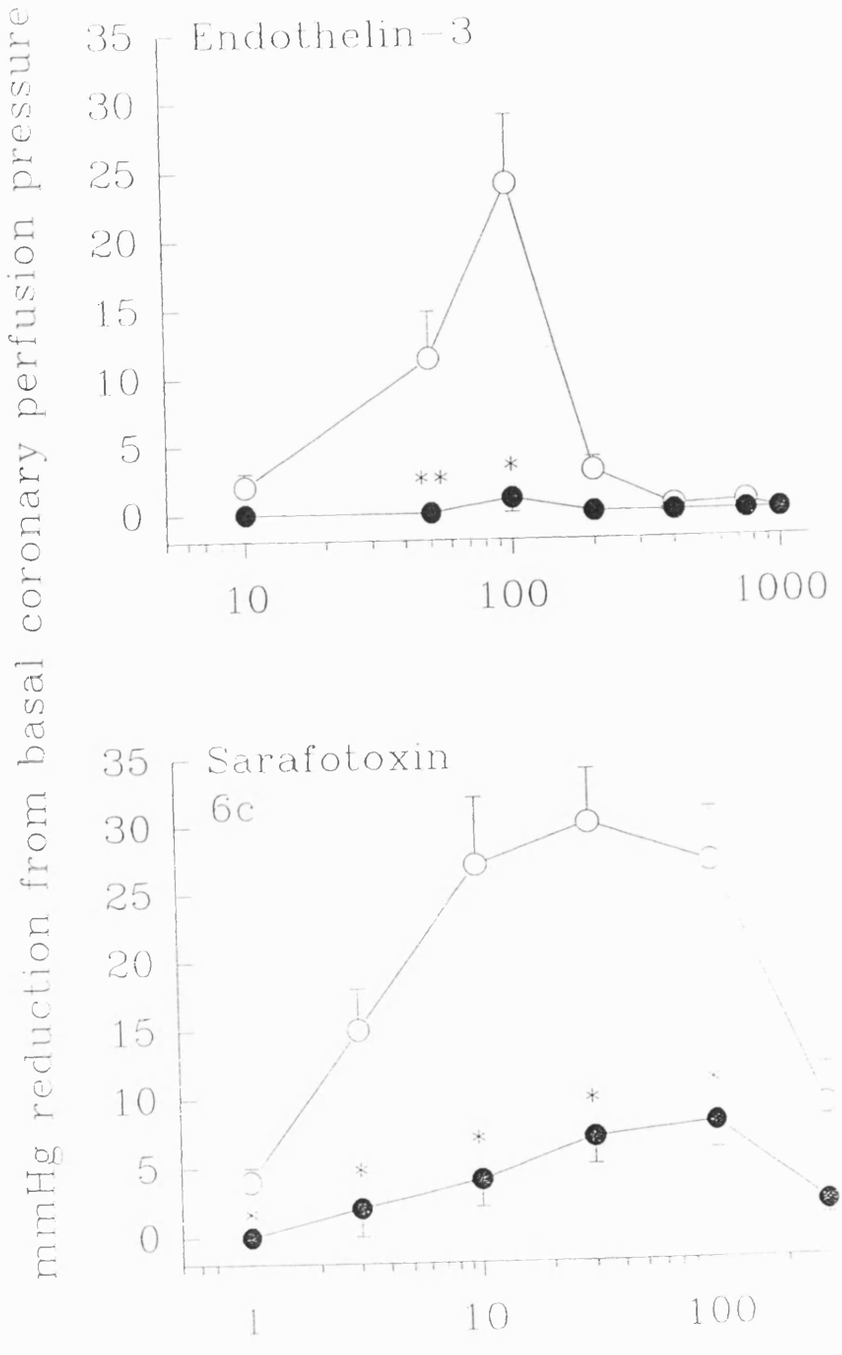


Figure R-6

The initial coronary dilator component of the response to endothelin-3 and sarafotoxin 6c (pmol) is shown as reduction from basal perfusion pressure. Mean vasodilatation (± SEM, n=4-5) in time-matched control hearts (o-o) is compared with that following ischaemia / reperfusion (●-●). The attenuation of vasodilatation in ischaemic / reperfused preparations achieves significance when analysed by Mann Whitney U-test, as denoted by * ($P<0.05$) and ** ($P<0.01$).

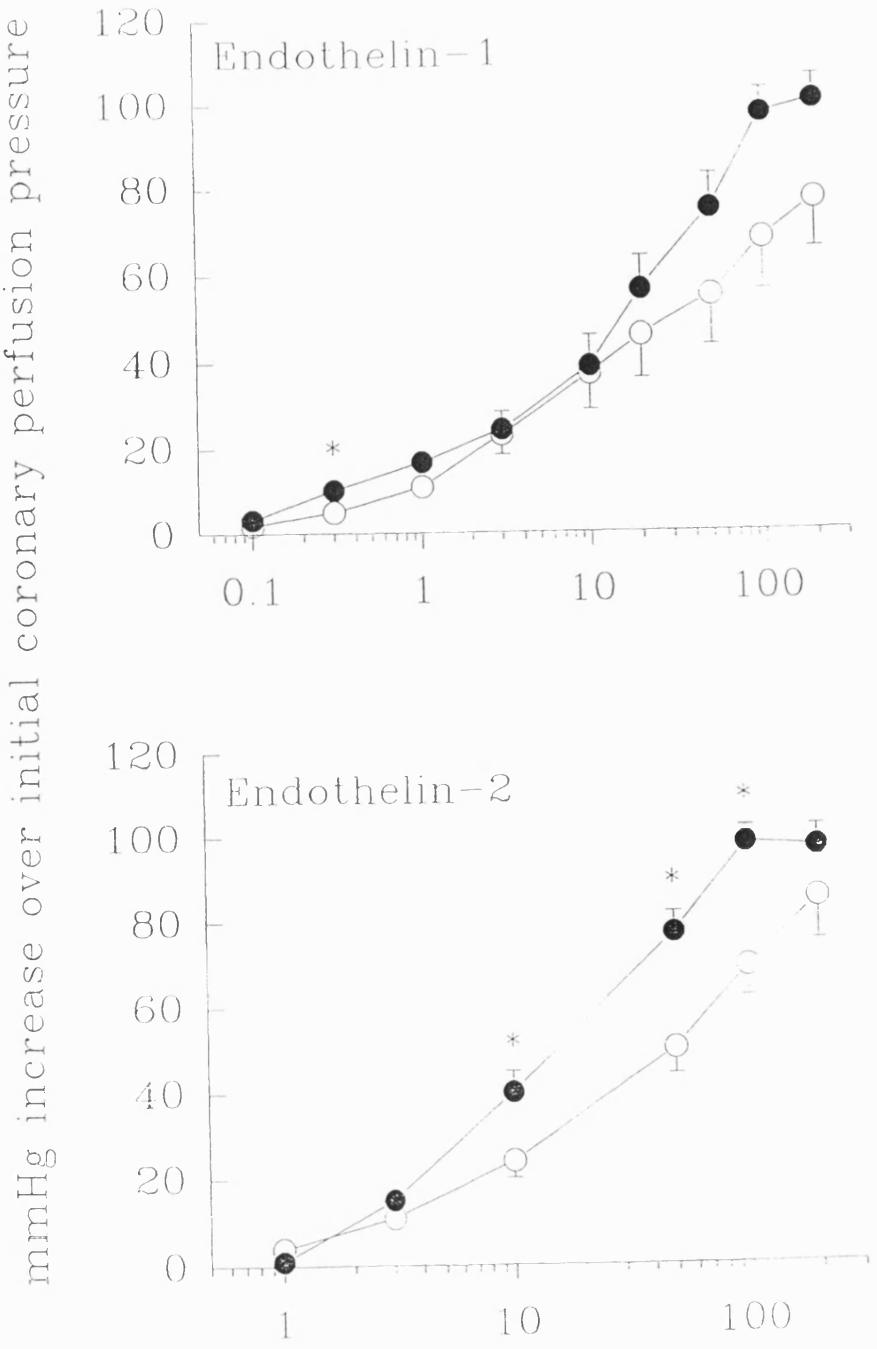


Figure R-7

The cumulative responses elicited by endothelin-1 and endothelin-2 (pmol) in control perfused (o-o) and ischaemic / reperfused preparations (●-●). Responses are from the same hearts as seen in figure R-5 (mean ± SEM, n=5-6). The enhancement at some doses achieves statistical significance as denoted by * ($P<0.05$) when analysed by Mann Whitney U-test.

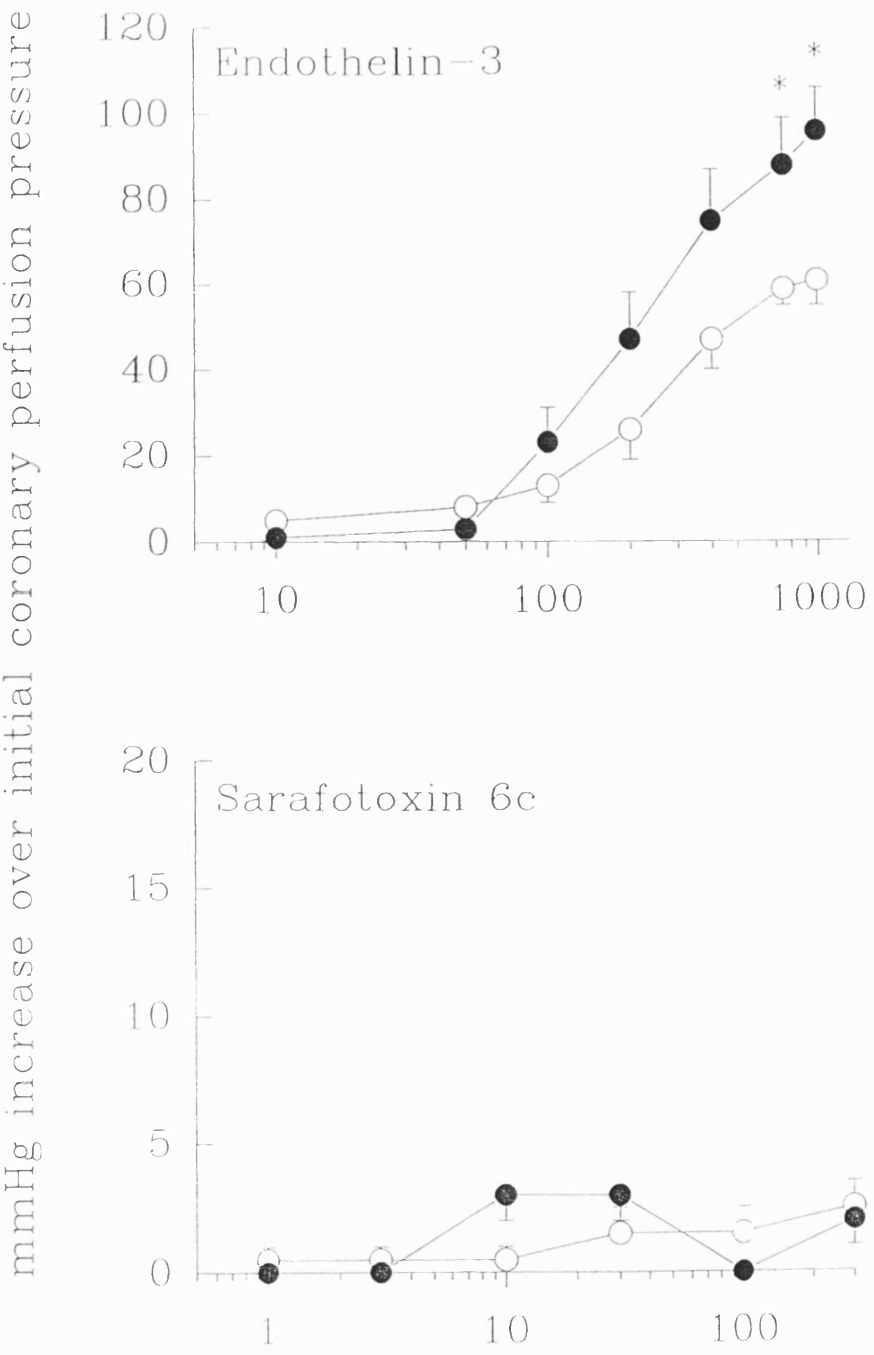


Figure R-8
Vasoconstriction elicited by injection of endothelin-3 and sarafotoxin 6c (pmol) is shown as mean ± SEM (n=4-5, same preparations as for figure R-6) increases above initial perfusion pressure (NB. Different *y* axis scale for sarafotoxin 6c). The responses in time-matched control preparations (o-o) are compared with those in ischaemic / reperfused hearts (●-●) by Mann Whitney U-test. Statistical significance is donated as asterisks above the relevant dose as * ($P<0.05$).

5.3 Comparison of the effects of ischaemia and reperfusion on the responses to the endothelin peptides and sarafotoxin 6c, with effects on responses to other vasoactive substances

5.3.1 Coronary vasodilator agents

As the vasodilator component of the endothelin response was affected by this ischaemia / reperfusion protocol, other vasodilator agents were similarly examined. This was done to verify whether the vasodilator loss was selective for endothelins.

Figures R-9 to R-11 show the effect of ischaemia / reperfusion on responses to non-endothelin coronary dilator agents. The responses to adenosine, bradykinin, papaverine and sodium nitroprusside are attenuated following ischaemia / reperfusion when compared with time-matched controls. The reduction in the verapamil response (figure R-11) is smaller in contrast to the effect on other agents.

5.3.2 Coronary vasoconstrictor agents

The selectivity of the vasoconstrictor enhancement seen with the endothelins was investigated by use of two different vasoconstrictors, phenylephrine and Bay K 8644. Responses to these agents in this preparation are shown in figure R-12. There is a slight inhibition of the vasoconstriction evoked by these agents after ischaemia and reperfusion which achieves statistical significance at some of the lower doses.

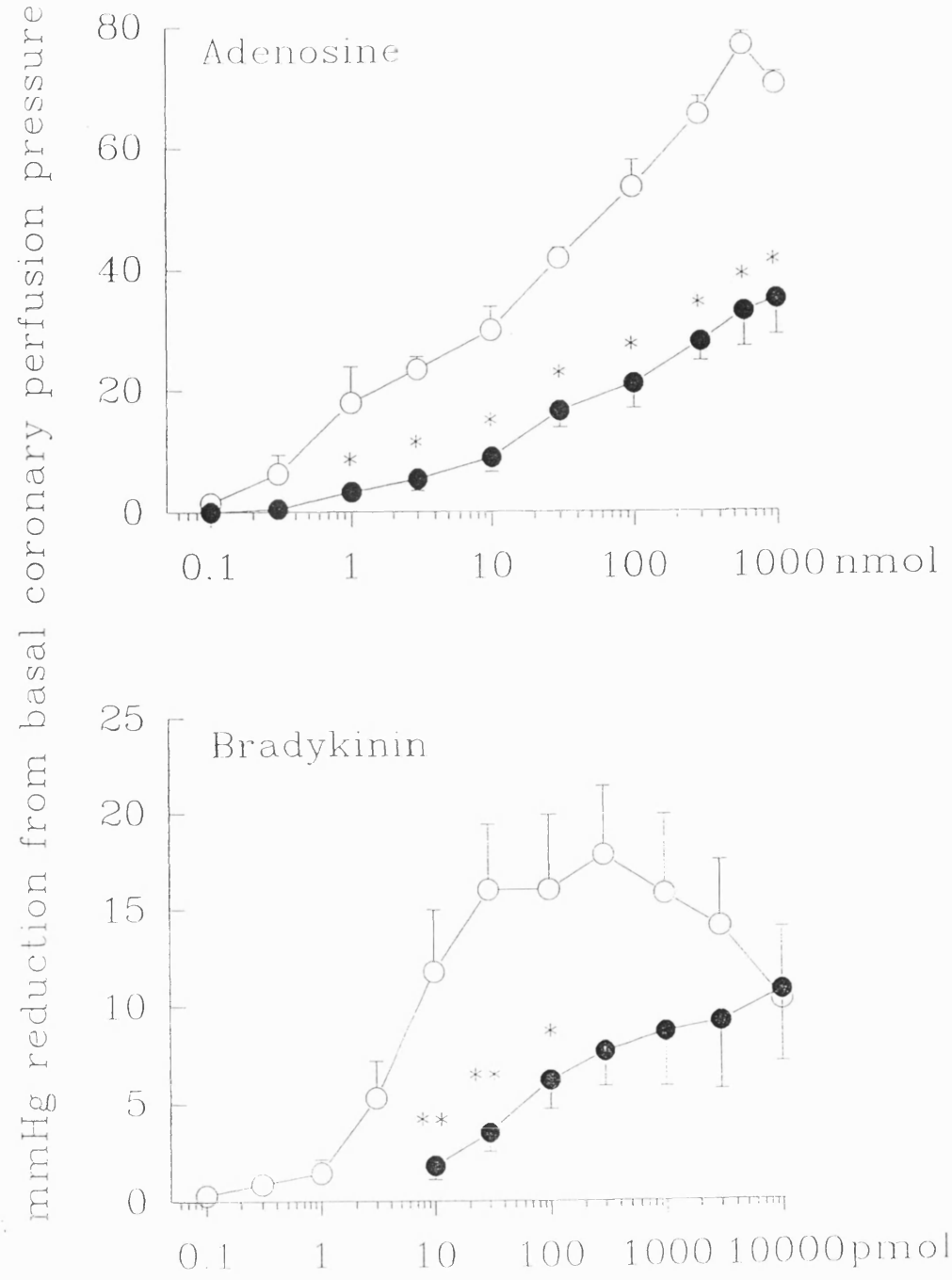


Figure R-9

The mean (± SEM) coronary dilator responses to adenosine (n=4) and bradykinin (n=6-12) are shown above as reduction from basal perfusion pressure. Responses in time-matched controls (o-o) are compared with those in ischaemic / reperfused preparations by Mann Whitney U-test. Statistical significance is denoted as asterisks above the relevant dose as * ($P<0.05$) or ** $P<0.01$).

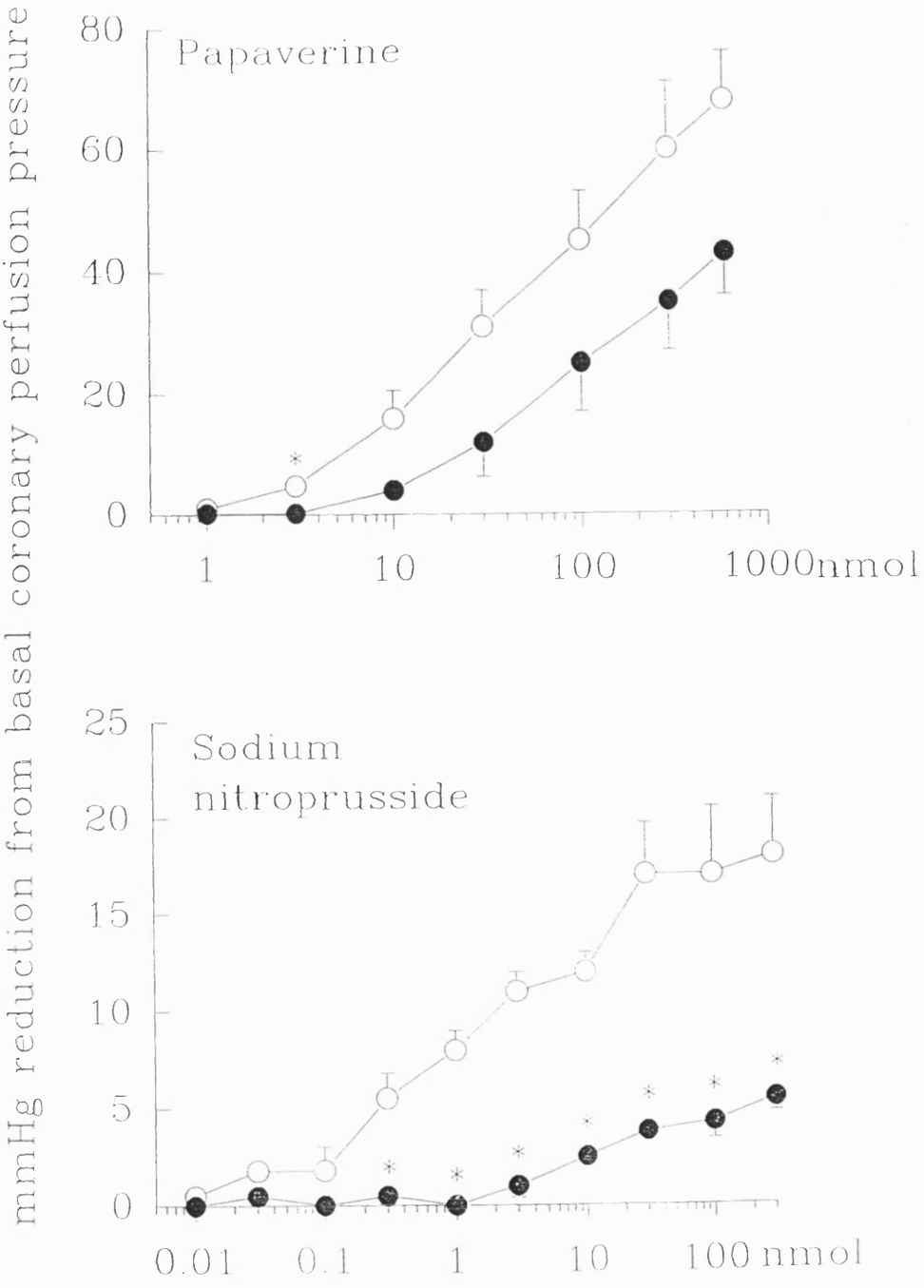


Figure R-10

The mean (± SEM) coronary dilator responses to papaverine and sodium nitroprusside (n=4 for both graphs) are shown as reduction from basal perfusion pressure. Responses in time-matched control hearts (o-o) are compared with those in ischaemic / reperfused preparations (●-●) by Mann Whitney U-test. Statistical significance is denoted as an asterisk above the relevant dose as * ($P<0.05$).

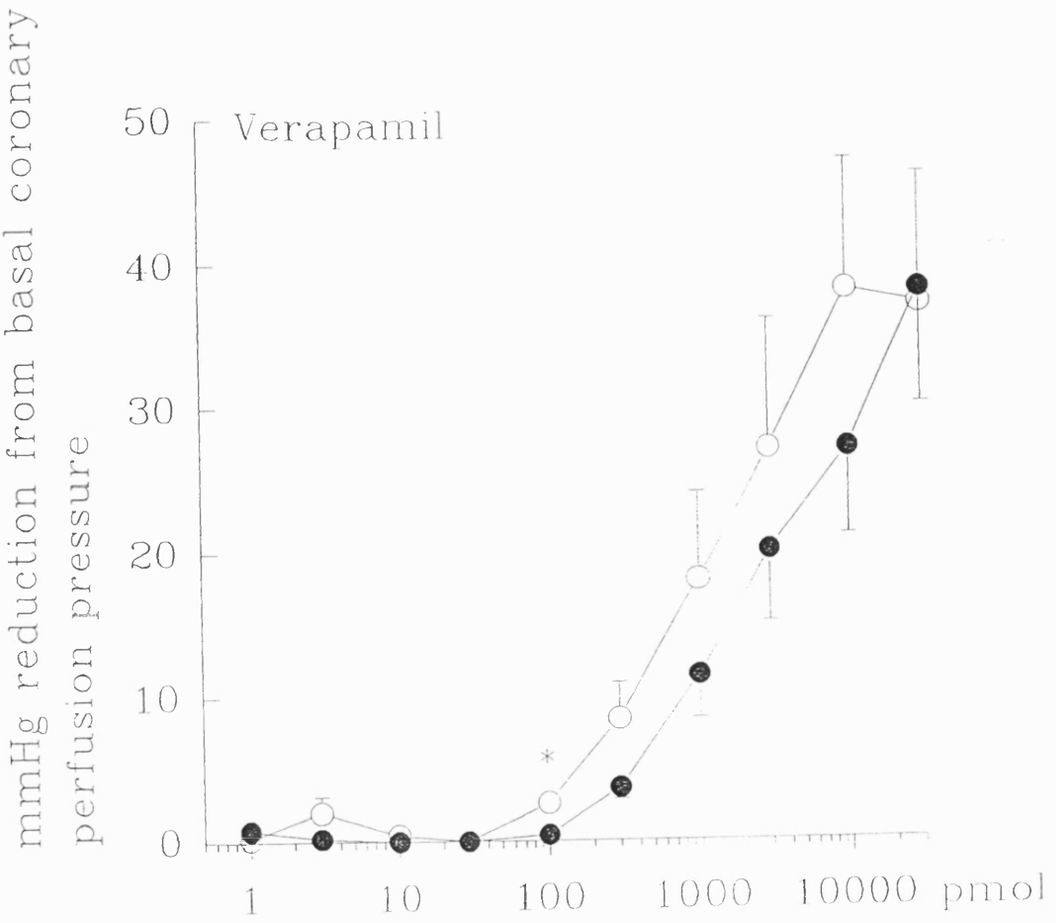


Figure R-11
The coronary dilator responses to verapamil are shown above. Mean (± SEM, n=4) responses are shown as reduction from basal perfusion pressure in control hearts (o-o) and ischaemic / reperfused preparations (●-●), and compared using Mann Whitney U-test. Statistical significance is denoted as * ($P<0.05$).

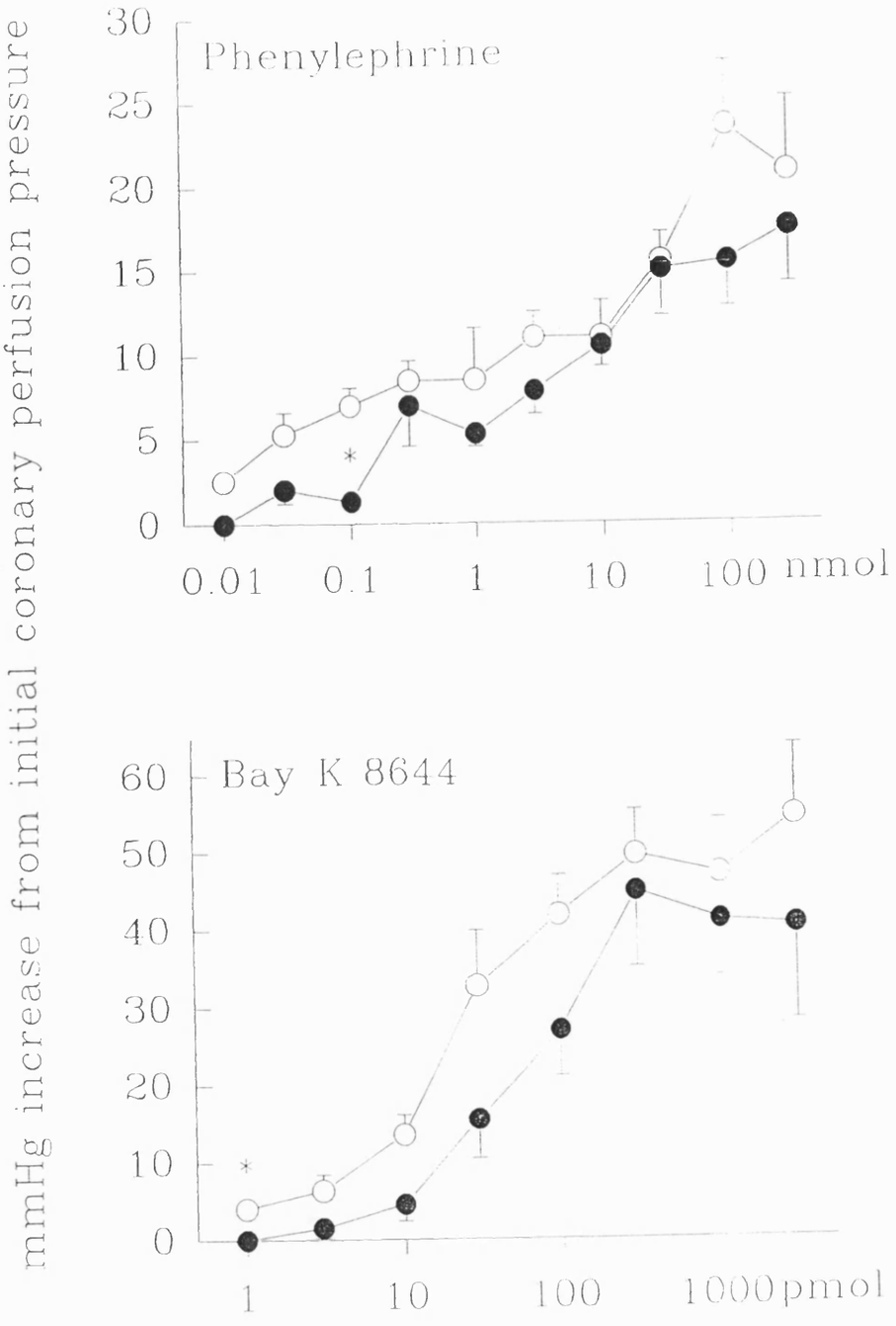


Figure R-12

The vasoconstrictor responses elicited by phenylephrine and Bay K 8644 in isolated perfused rat hearts are shown in the above figure. Mean (± SEM, n=4) responses are shown in control perfused (o-o) and ischaemic / reperfused (●-●) preparations. Where the attenuation of the responses achieves statistical significance by Mann Whitney U-test, this is denoted by * ($P<0.05$).

5.4 Effect of indomethacin and L-NMMA on vasodilator responses to endothelin-3, compared with the effect on other coronary vasodilators

The enhancement of the vasoconstrictor response to the endothelins following ischaemia / reperfusion could simply be due to a loss of the vasodilator component of the response. In order to investigate the effects of vasodilator loss, cyclooxygenase and nitric oxide synthase inhibitors were used to attenuate the vasodilator component of the response to endothelin-3, in the absence of ischaemia / reperfusion. Combinations of other NO synthase inhibitors (N^G-nitro L-arginine methyl ester and N^G-nitro L-arginine, both 100μM) with indomethacin (10μM) in preliminary experiments were ineffective in eliciting any inhibition of either the thrombin- or the bradykinin-induced vasodilatation. Hence L-NMMA was used in combination with a similar concentration of indomethacin. A dose of endothelin-3 was selected which had a sub-maximal vasodilator effect but which caused minimal vasoconstriction (see figures R-6 and R-8). The effects of these inhibitors on coronary vasodilatation to bradykinin and thrombin were compared with those on endothelin-3. The combined inhibitory agents L-NMMA (100μM) and indomethacin (10μM) had no significant effect on basal perfusion pressure ($P>0.05$) measured at the time of addition of coronary dilators.

The mean coronary vasodilator responses to bradykinin and thrombin were reduced by 74% and 72% respectively from control values (figure R-13), suggesting that the concentrations used were sufficient to attenuate the effect of nitric oxide synthase or cyclooxygenase products (without distinguishing which was the effector).

In control hearts, the mean vasodilator effect of a sub-maximal (50pmol) dose of endothelin-3 was 22 ± 2 mmHg. There was, however, a wide degree of variability in the post-inhibitor response to endothelin-3 (from 0 to 27 mmHg vasodilatation). Hence, although a trend toward an inhibitory effect from these combined agents was seen this was not statistically significant. Therefore, the inhibition of neither NO synthase or cyclooxygenase could be considered responsible for the abolition of the vasodilator phase of the endothelin response. Perfusion of the combined inhibitors did not result in any

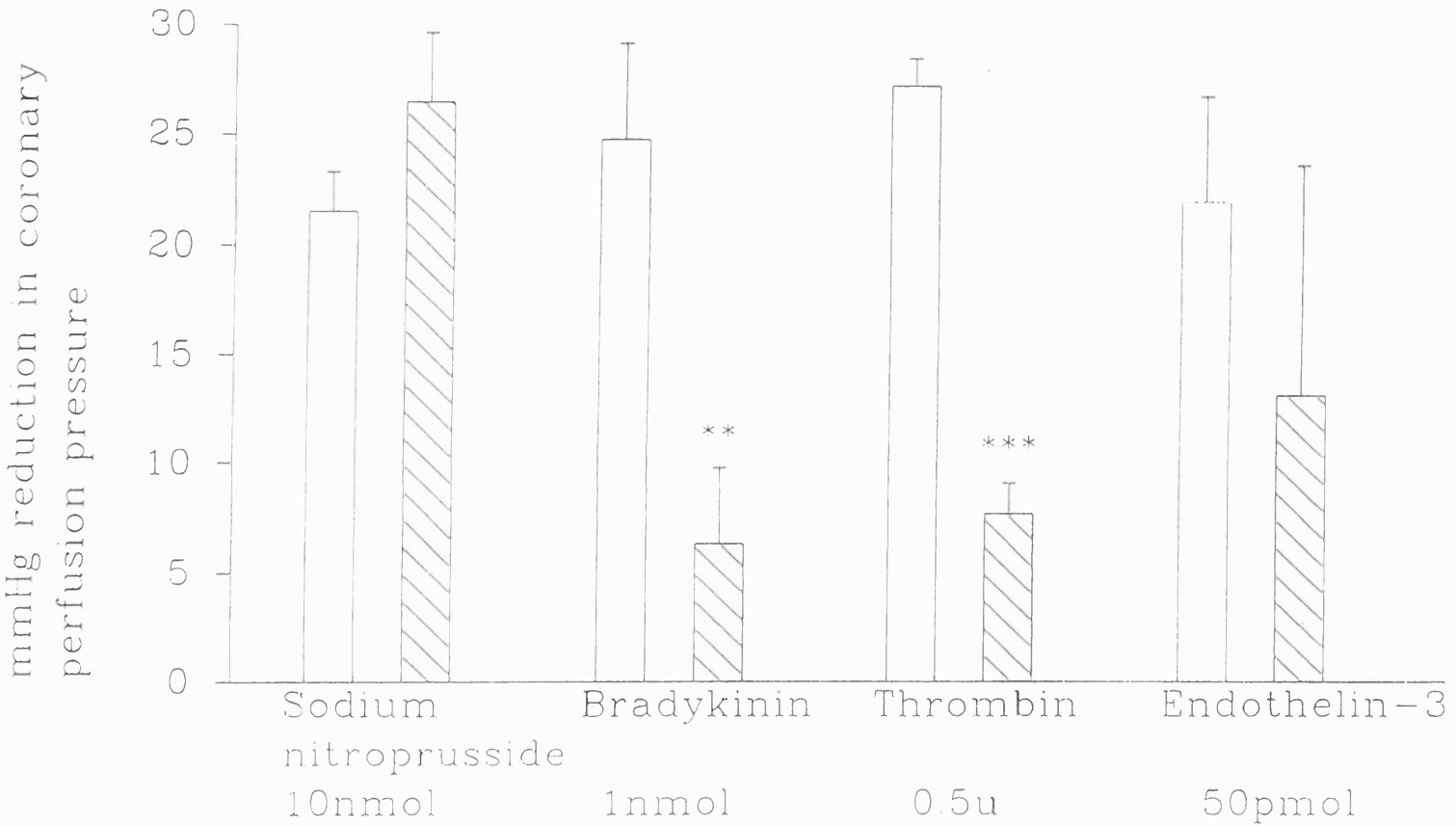


Figure R-13

Mean (± SEM, n=4-6) coronary dilator responses to sodium nitroprusside and bradykinin and the vasodilator phase of the response to thrombin are compared with the coronary dilator phase of the response to 50pmol endothelin-3. Open bars denote means from control preparations, and hatched bars give data from preparations perfused using a combination of indomethacin (INDO, 10μM) and N monomethyl-L-arginine (L-NMMA, 100μM). The response to sodium nitroprusside is unchanged following combined INDO / L-NMMA perfusion, whereas responses to bradykinin and thrombin are significantly attenuated (denoted by ** ($P<0.01$) and *** ($P<0.001$). Coronary dilatation to endothelin-3 appears to be attenuated but this does not achieve significance.

significant enhancement of vasoconstriction to the endothelin-3 dose selected, when compared using Mann Whitney U-test.

5.5 Effect of glibenclamide on responses to endothelin-3

Another mechanism of vasodilatation investigated, was the ATP-dependent potassium current (Lippton *et al.*, 1991). Perfusion of 10μM glibenclamide to block this current had the effect of increasing basal perfusion pressure by a mean value of 20 ± 8 mmHg (mean ± SEM, n=4) whereas vehicle alone had no effect on coronary perfusion pressure ($P>.0.05$). This makes the interpretation of changes in the vasodilatation response difficult.

Following glibenclamide infusion, the vasodilator responses to a range of doses of endothelin-3 were enhanced, presumably as a result of the increased basal perfusion pressure (figure R-14a). However, despite the difference in perfusion pressure, the vasodilator response to the potassium channel opener lemakalim was almost completely abolished (figure R-14b) suggesting that the concentration of glibenclamide used was sufficient to overcome any vasodilatation due to opening of the ATP-dependent potassium channel, and that other mechanisms were responsible for the vasodilatation to endothelin-3. However, it is not possible to eliminate a partial contribution to the vasodilatation from an ATP-dependent potassium current as a reduction would have been masked by the enhancement in vasodilatation resulting from increased basal perfusion pressure.

In contrast, the vasoconstrictor phase of the endothelin-3 response was attenuated after glibenclamide perfusion, this being a significant effect at the 20, 100 and 200pmol doses ($P<0.05$ by Mann Whitney U-test; see figure R-14c).

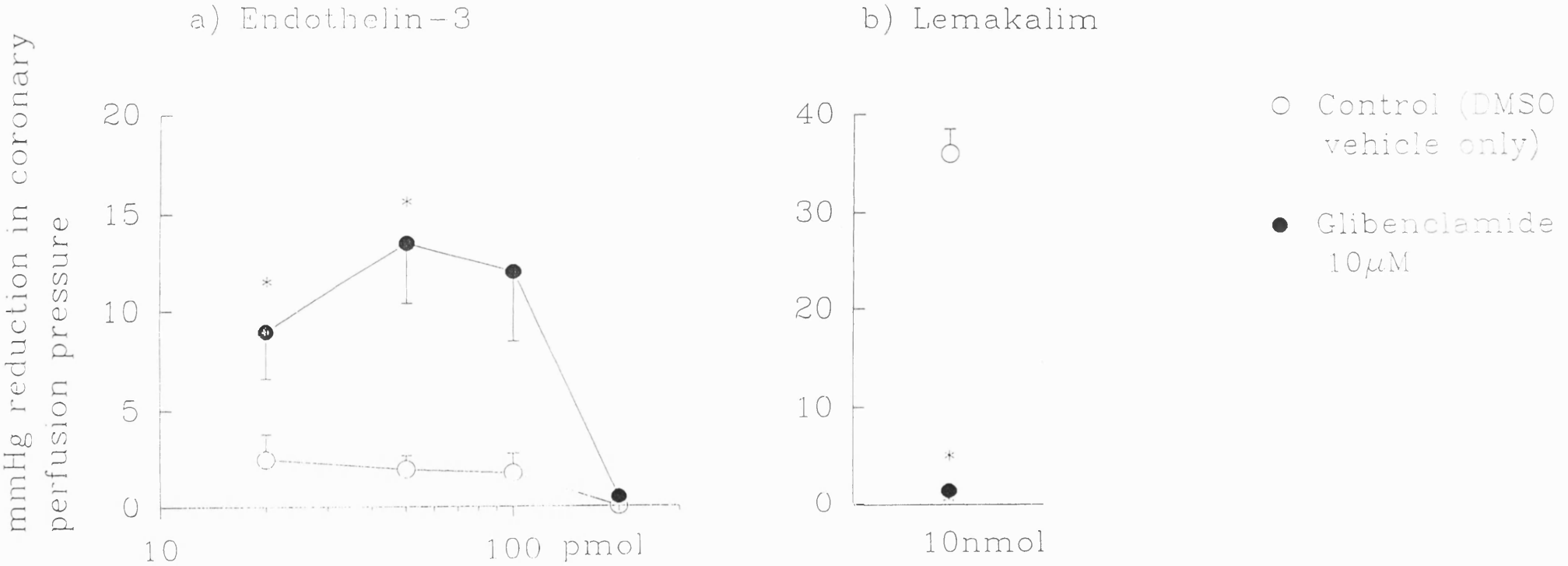


Figure R-14 a&b

The vasodilator phase of the endothelin-3 response in isolated perfused rat hearts (a) is compared here in DMSO vehicle control (o-o) and in (10μM) glibenclamide-perfused hearts (●-●). Figure R-14b shows the effect of the glibenclamide perfusate on a response to a submaximal dose of lemakalim. Mean (± SEM) responses are shown as reduction from basal perfusion pressure which was 79 ± 4mmHg in control hearts and 110 ± 11 mmHg in glibenclamide-perfused hearts (n=4). Statistical significance (Mann Whitney U-test) is denoted as * ($P<0.05$).

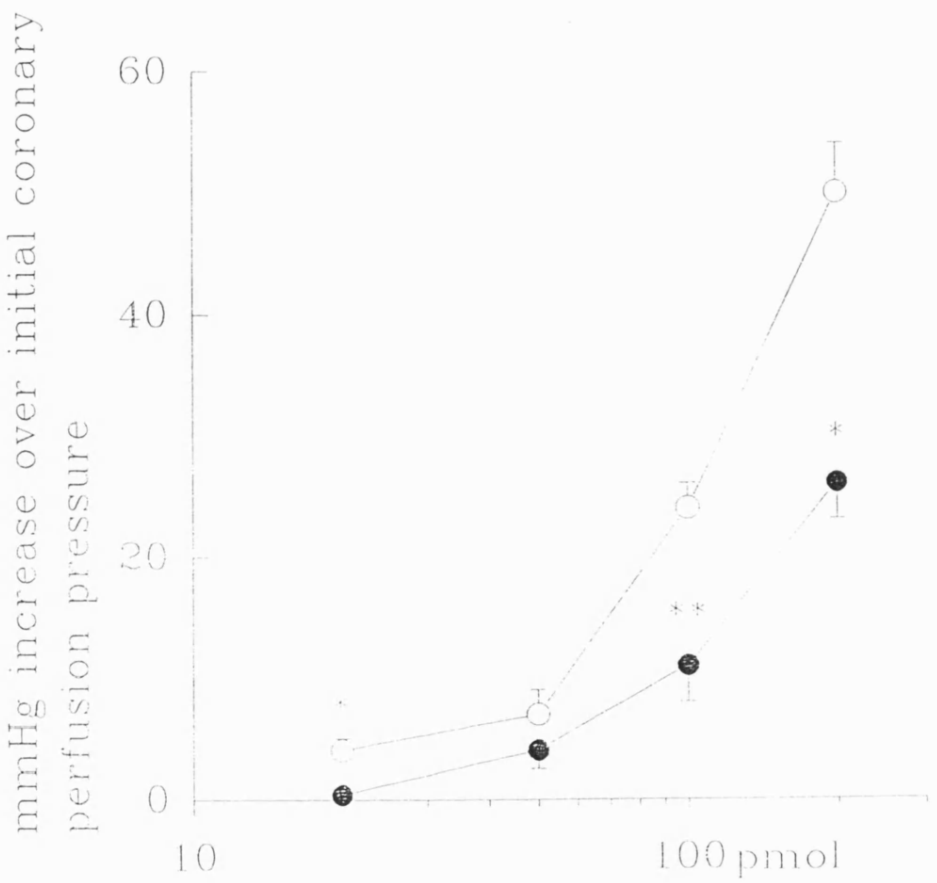


Figure R-14c
The effect of glibenclamide perfusion on the vasoconstrictor response to endothelin-3 in the same hearts as shown in R-14a. Responses are shown as mean (± SEM, n=4) cumulative increase from basal perfusion pressure and controls (o-o) are compared with glibenclamide perfused preparations (●-●) using Mann Whitney U-test. Statistical significance is denoted by * ($P<0.05$) and ** ($P<0.01$).

5.6 Effect of perfusion of BQ-123 on responses to endothelin-1 and endothelin-3

The receptor subtype involved in the vasoconstrictor and vasodilator phases of the endothelin peptide responses was investigated, initially using an ET_A receptor antagonist, BQ-123 (Ihara *et al.*, 1991). This agent has been shown to be highly selective for ET_A receptors but to have no effect on coronary vasoconstriction to potassium chloride, noradrenaline, prostaglandin $F_{2\alpha}$, acetylcholine or histamine (Ihara *et al.*; *ibid*).

Perfusion of 1μM BQ-123 had no significant effect on basal perfusion pressure (75 ± 5 mmHg) after 20 minutes' perfusion, when compared with time-matched control (82 ± 4 mmHg, $P>0.05$ by Student's t-test, n=9-10). This concentration of BQ-123 was sufficient to cause a significant antagonism of the vasoconstrictor responses to both endothelin-1 and endothelin-3, (figure R-15) when compared with controls using Mann-Whitney U-test. However, the magnitude of the vasodilator response to both endothelins was unaffected in the same experiments (figure R-16). Following BQ-123 perfusion, the morphology of the endothelin-1 responses at doses where vasoconstriction was abolished (10-100pmol endothelin-1) more closely resembled that of sarafotoxin 6c, the ET_B-selective agonist (see figure R-17). The duration of the vasodilatation to endothelin-1 was increased by BQ-123 perfusion when compared with controls and this difference gained statistical significance at one point on the dose-response curve ($P<0.01$, n=5-6; figure R-18a). The duration of the vasodilator effect of endothelin-3 was not significantly changed by BQ-123, ($P>0.05$ at all doses, n=4; figure R-18b), and this may reflect the less potent vasoconstrictor effect of this isopeptide in the range of its vasodilator effect.

In addition to this evidence for different receptors mediating the two phases of endothelin responses, a preliminary study testing the selectivity of sarafotoxin 6c for ET_B receptors showed that 2μM BQ-123 did not affect the response to a 10pmol dose of the peptide compared with a parallel control. This concentration of BQ-123 resulted in complete inhibition of the vasoconstrictor response to a subsequent injection of endothelin-1 (50pmol) in the same experiment, this dose subsequently eliciting a prolonged vasodilatation

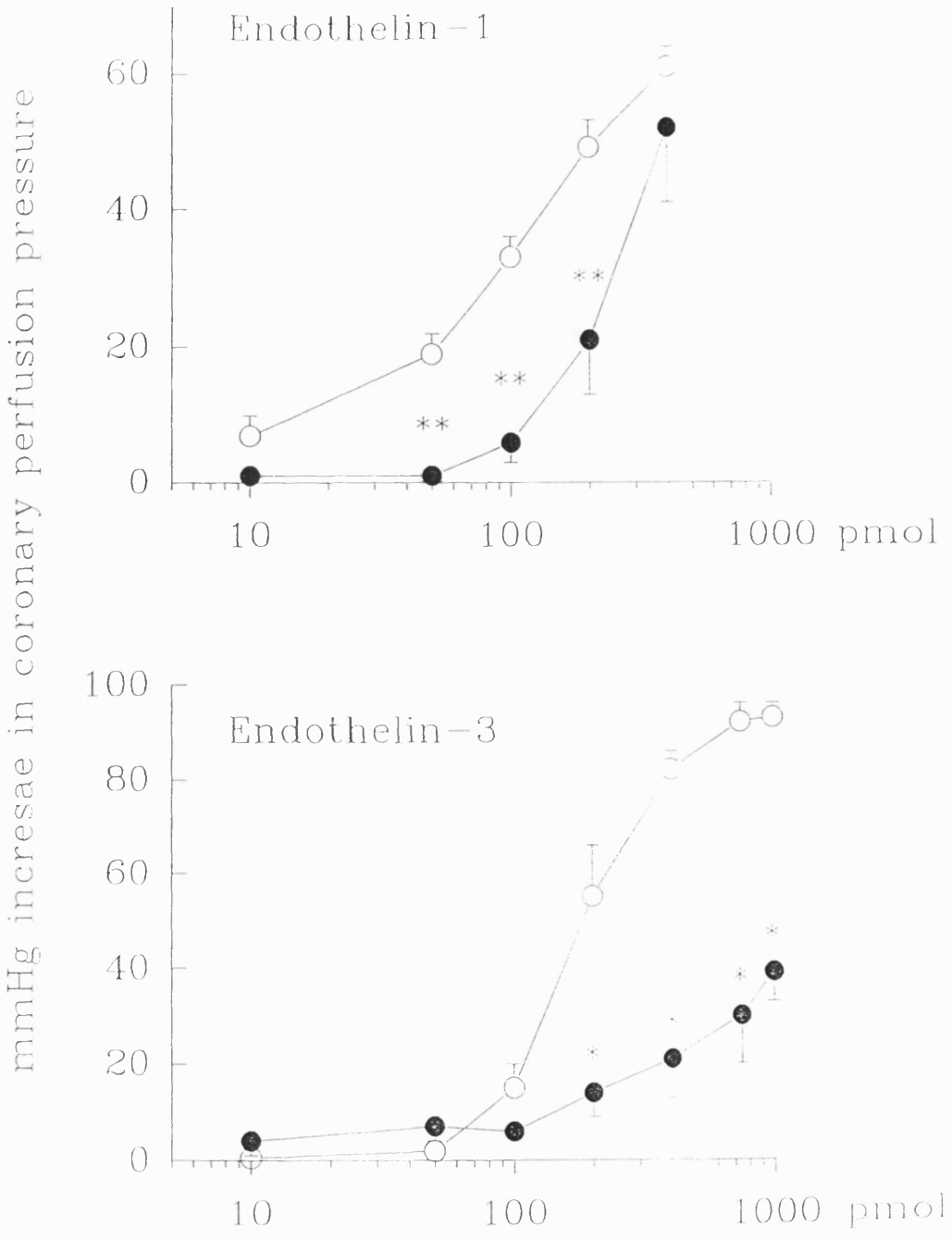


Figure R-15
Coronary constrictor responses elicited by endothelins -1 and -3 in perfused rat hearts. Control responses (o-o, n=6) are compared with responses following 20 minutes' perfusion with 1μM BQ-123 (●-●, n=5). Statistical significance in the attenuation of vasoconstriction is denoted by * ($P<0.05$) and ** ($P<0.01$) as determined by Mann Whitney U-test.

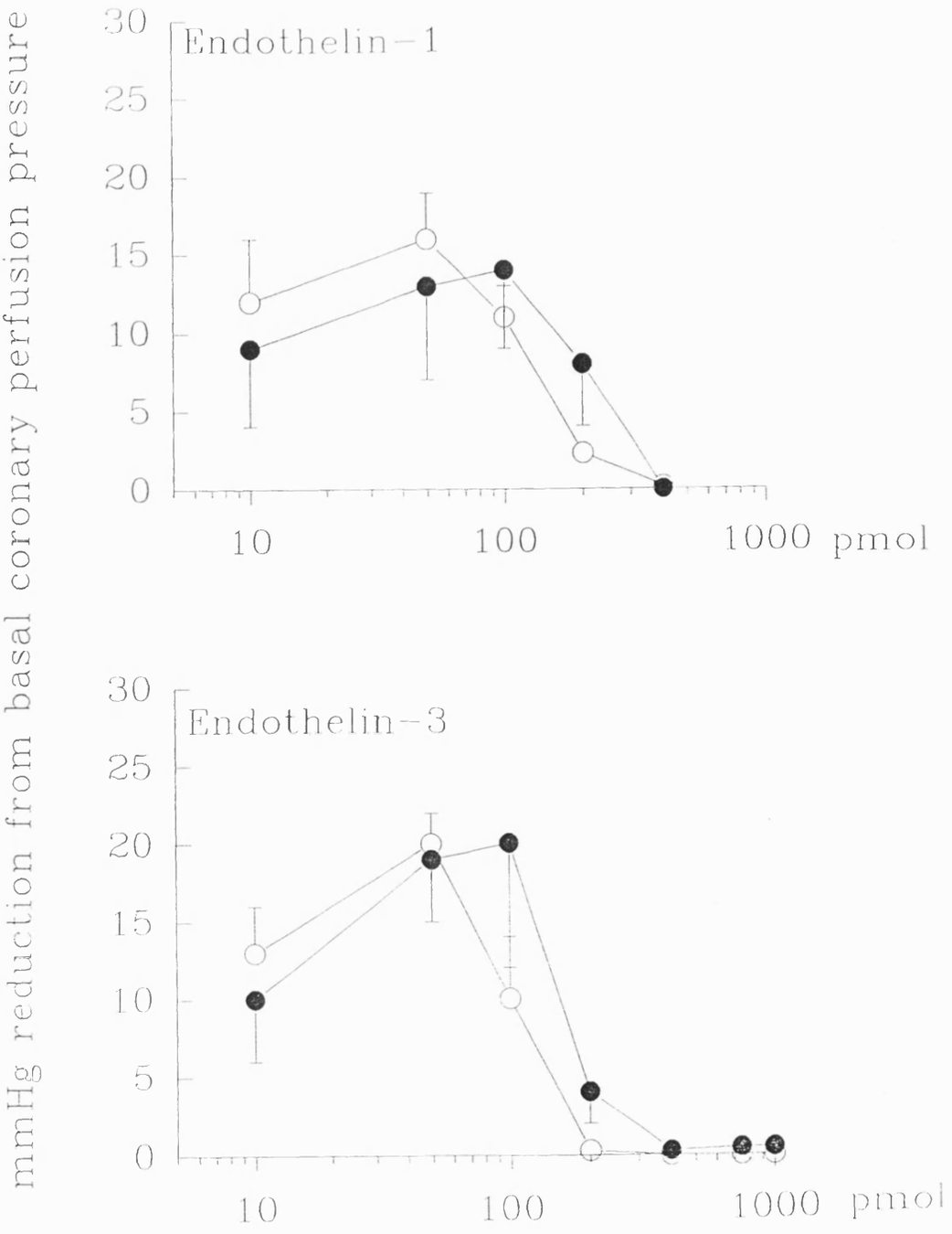


Figure R-16
Coronary dilator responses elicited by endothelins -1 and -3 in perfused rat hearts in the same preparations as figure R-15. The control responses (o-o, n=6) are compared with responses after 20 minutes' perfusion with 1μM BQ-123 (●-●, n=5). There are no significant differences in the magnitude of the dilator responses to either peptide following BQ-123 perfusion (Mann Whitney U-test).

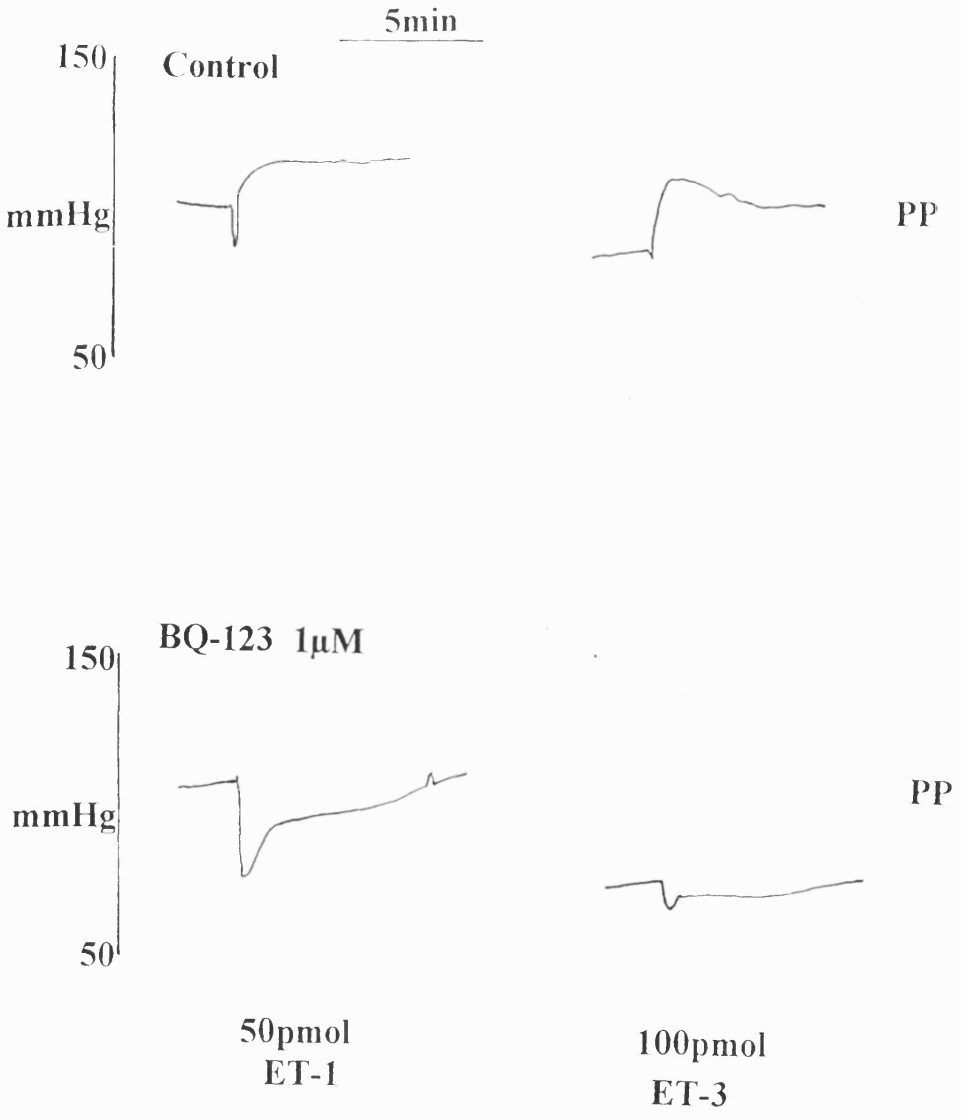


Figure R-17
Examples of the changes in perfusion pressure (PP) in response to endothelin-1 (ET-1, left) and endothelin-3 (ET-3, right), comparing controls (upper traces) with responses following 20 minutes' perfusion with BQ-123 (1μM, lower traces).

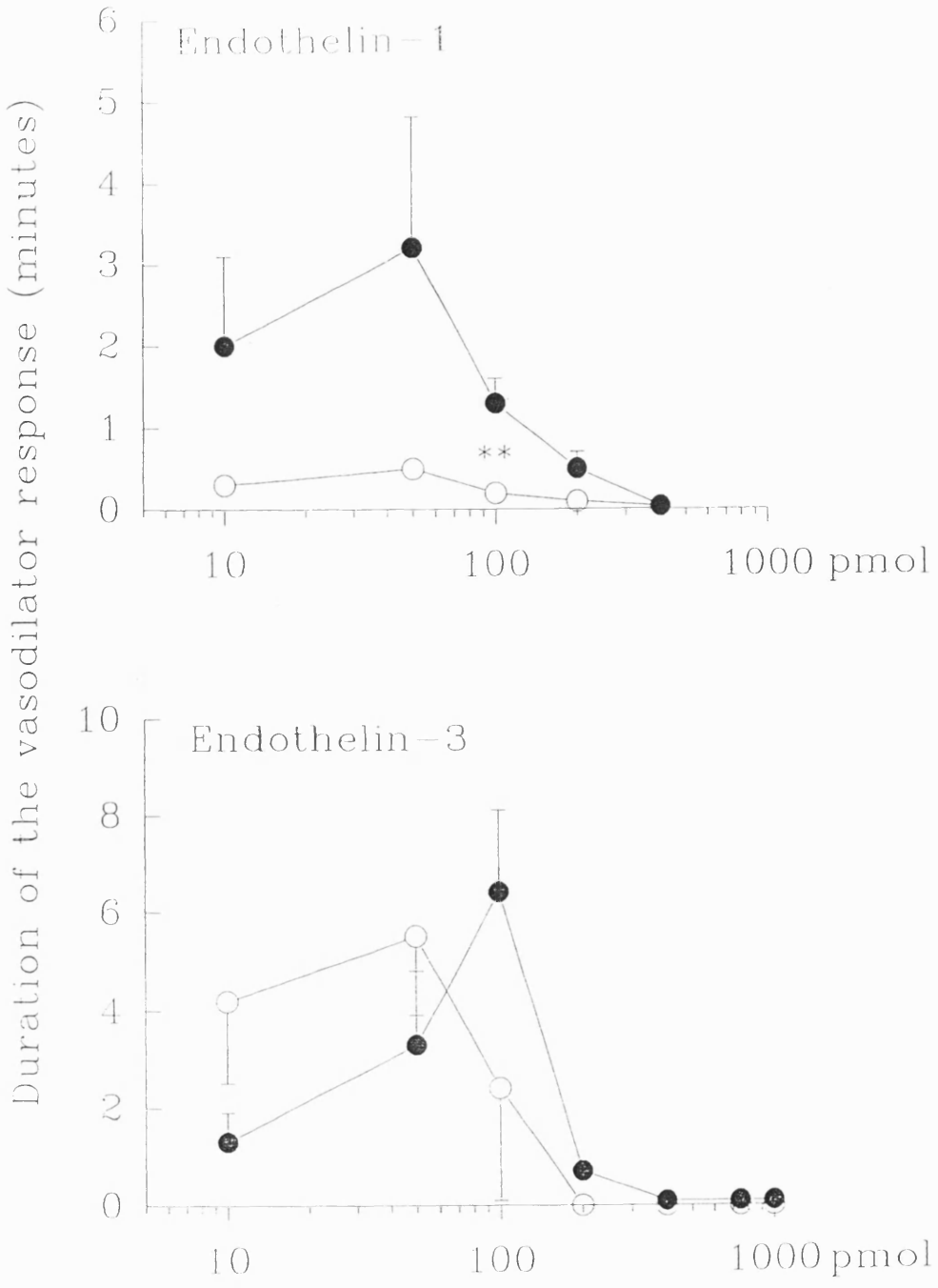


Figure R-18

The above figure shows the duration of the coronary dilator response (minutes from onset of vasodilatation to recovery of the initial perfusion pressure) in response to bolus injection of endothelins -1 and -3 in the same preparations shown in figures R-15 and R-16. The mean (± SEM, n=5-6) duration of response in control hearts (o-o) is compared to that following perfusion of BQ-123 (μM, ●-●). The significant increase in duration of the response to 100pmol endothelin-1 in BQ-123 perfused hearts is denoted by ** ($P<0.01$, Mann Whitney U-test).

of 16mmHg. In the control heart however, the endothelin-1 response was purely vasoconstrictor (20mmHg magnitude).

5.7 Effect of ET_B receptor desensitisation with sarafotoxin 6c, on vasodilator responses to endothelin-1 and comparison with other coronary vasodilator and vasoconstrictor agents

Following three 100pmol doses of sarafotoxin 6c repeated at 10 minute intervals, the vasodilator phase of the endothelin-1 response was completely inhibited ($P<0.01$, $n=6$, figure R-19a). The inhibitory effect was selective for endothelin-1 as the responses to other coronary vasodilator agents tested (sodium nitroprusside and bradykinin) were unchanged after desensitisation ($P>0.05$, $n=4$). However, the vasoconstrictor phase of the endothelin-1 was enhanced following desensitisation of the ET_B receptor with sarafotoxin 6c, the enhancement being of a magnitude similar to the degree of vasodilator loss (figure R-19b). This enhancement was again confined to the endothelin responses as the phenylephrine and Bay K 8644 constrictor responses were unaffected ($P>0.05$, $n=4$).

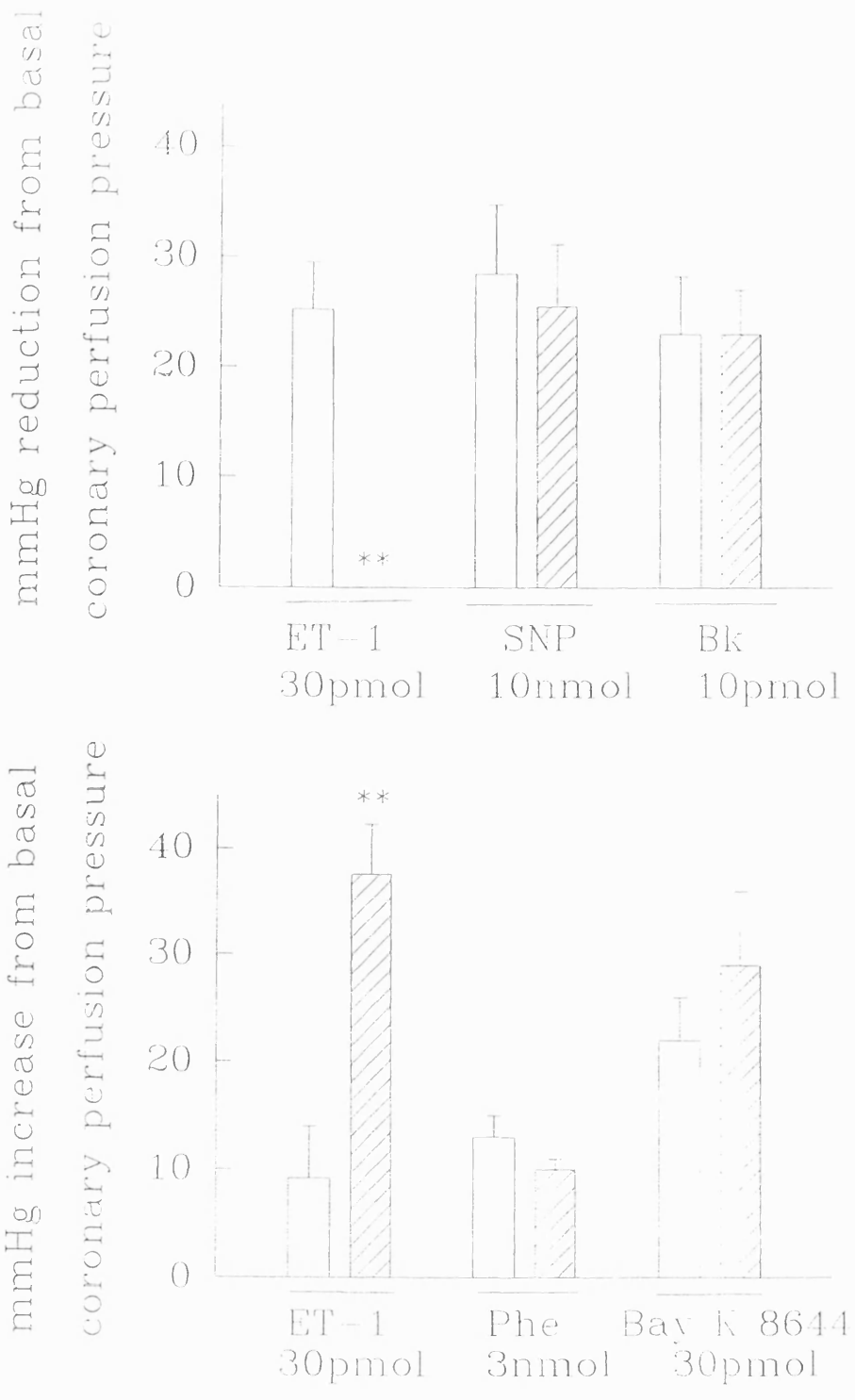


Figure R-19
Effects of ET_B receptor desensitisation, using 3x100pmol doses of sarafotoxin 6c, on coronary dilator responses to endothelin-1, sodium nitroprusside and bradykinin (a). Effects of desensitisation on coronary constrictor responses to endothelin-1, phenylephrine and Bay K 8644 are shown in the lower figure (b). Controls (open bars) are compared with desensitised preparations (hatched bars) using Mann Whitney U-test, and significance is denoted by ** ($P<0.01$). n=6 for endothelin-1 responses and n=4 for all other agonists.

6.0 Cell signalling

6.1 Calcium responses to endothelin-1 using monolayers of cells in 96-well plates

Initial experiments used groups of fura-2-loaded cells observed under low power (20x quartz fluorescence objective) in 96 well plates. This provided the intracellular calcium concentration ($[Ca^{2+}]_i$) for an area of about 20 confluent cells per view in a monolayer. $[Ca^{2+}]_i$ thus recorded had a mean basal value of 73 ± 6 nM (n=4) calculated using a standard curve (see section 3.2.5.1).

Under these conditions the effects of endothelin-1 on $[Ca^{2+}]_i$ were established.

6.1.1 Effects of endothelin-1 on intracellular calcium concentration in coronary smooth muscle cell monolayers

Endothelin-1 (0.1nM to 10μM) produced a rise in $[Ca^{2+}]_i$ which was variable between cell cultures from different hearts. Despite standardisation of cell culture conditions, the peak calcium response to the 10μM concentration of endothelin-1 ranged from 160nM to 570nM, suggesting that the sensitivity of different cell cultures (from different hearts) was greatly variable (see section 6.1.2 below). Following the initial peak in $[Ca^{2+}]_i$, a more prolonged "plateau phase" of calcium increase was evident in most preparations and can be seen in response to a 10μM concentration of endothelin-1 in figure R-20. A concentration-response relationship is demonstrated in figure R-21a.

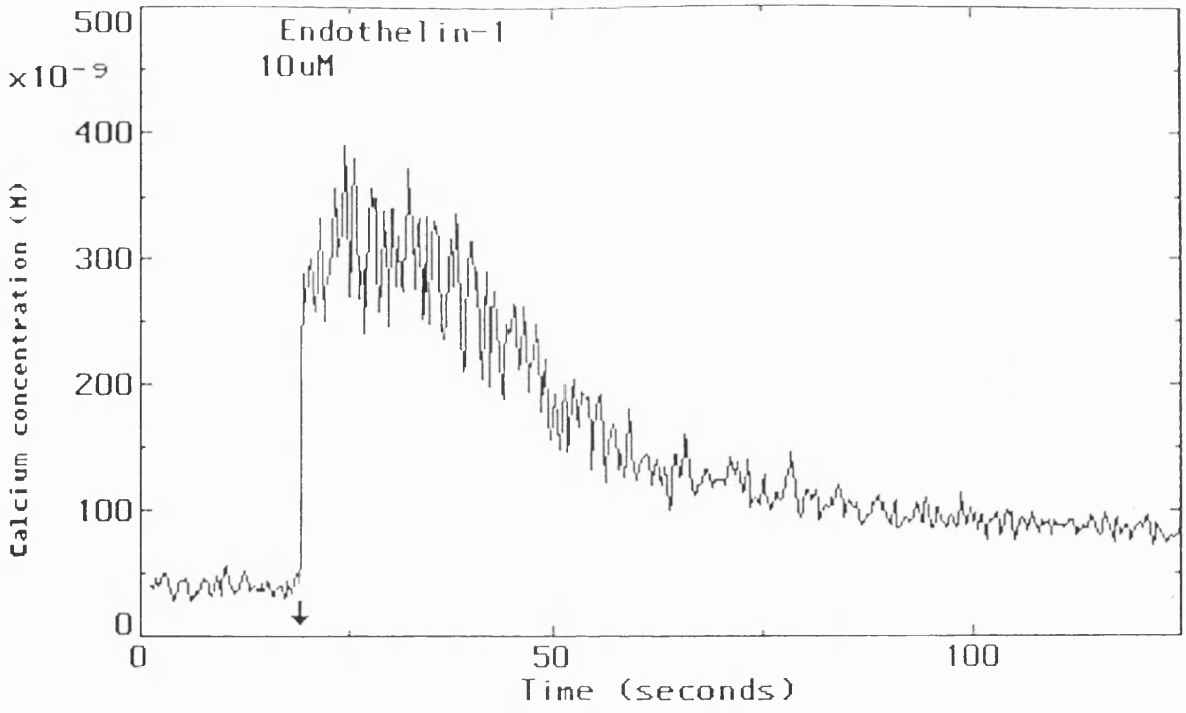


Figure R-20
A representative trace from a monolayer of porcine coronary smooth muscle cells, showing the increase in $[Ca^{2+}]_i$ to a 10μM concentration of endothelin-1 (added at arrow) which demonstrates the peak and subsequent plateau phases of the response

6.1.2 Effect of BQ-123 on calcium responses to endothelin-1 in coronary smooth muscle cell monolayers

Figure R-21 shows the increase in $[Ca^{2+}]_i$ to endothelin-1 (1nM-1μM) in a monolayer of coronary smooth muscle cells. The effect appears to be concentration-related and the initial response is inhibited almost completely when cells were preincubated for 3 minutes with 1μM BQ-123 (R-21b). The effect of the ET_A receptor antagonist BQ-123 (0.1-10nM) on the peak calcium response in these cells is summarised in figure R-22 which reveals inhibition at only the highest (10nM) BQ-123 concentration used, this appearing to be non-competitive. As the responses in cells from different primary cultures showed such variability, the individual experiments from which this summary is derived are illustrated in figure R-23. The trend for inhibition of the response by BQ-123 is seen in each of the cell preparations included, and the wide range of responsiveness is also apparent. However, these figures combined demonstrate that the endothelin-1 response is concentration dependent, and that the response is clearly inhibited by 10nM BQ-123. This inhibition is statistically significant ($P<0.05$) at higher endothelin concentrations.

The inhibition of endothelin-1 responses by BQ-123 was selective, as demonstrated by the lack of effect of this compound (1-100nM) on responses to a sub-maximal (1nM) concentration of bradykinin (figure R-24). There was no significant difference between means of the bradykinin responses in the presence or absence of BQ-123 ($P>0.05$, ANOVA).

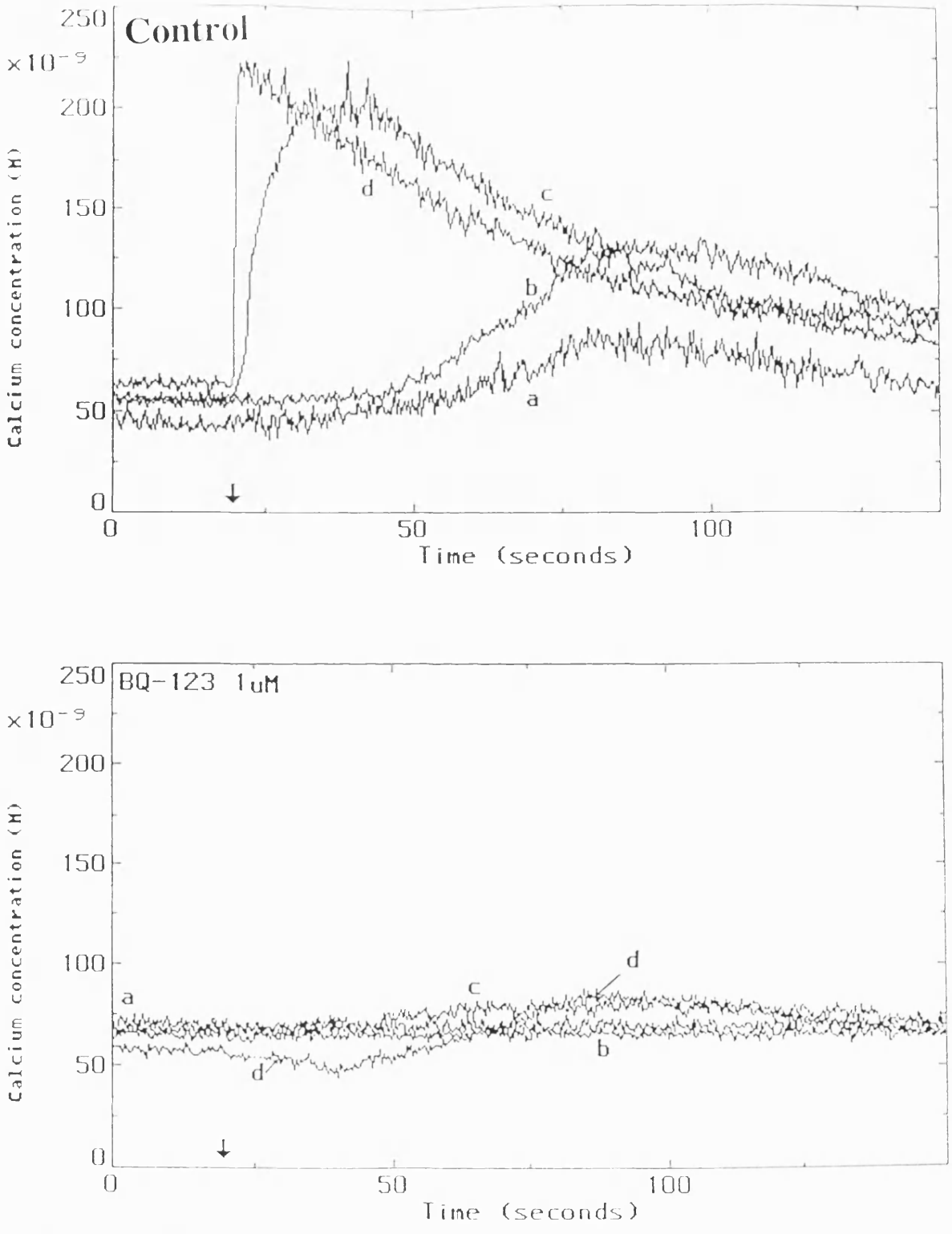


Figure R-21

The increases in $[Ca^{2+}]_i$ in porcine coronary smooth muscle cell monolayers, in response to 1nM (a), 10nM (b), 100nM (c),and 1μM endothelin-1 (added as indicated by the arrow). Each trace represents a separate group of cells from the same preparation. The upper panel shows responses in control hearts whereas the lower panel shows responses following 3 minutes' incubation with BQ-123 (1μM).

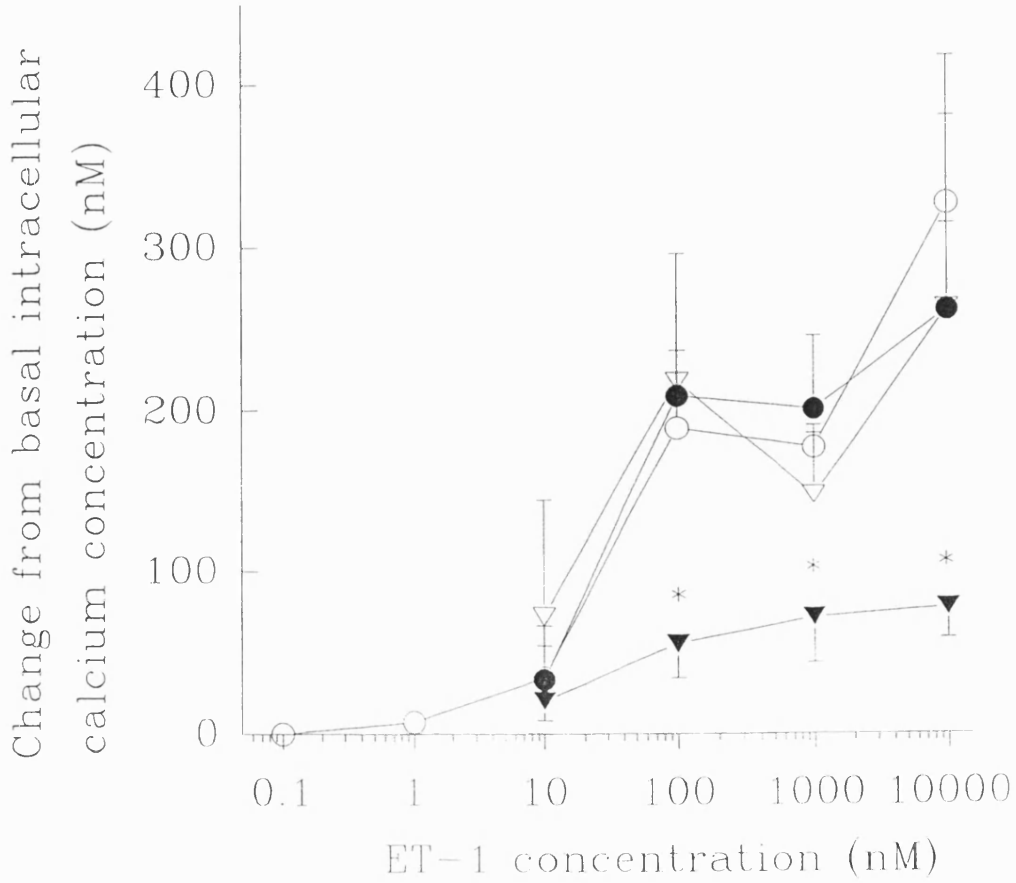


Figure R-22
Effects of endothelin-1 on $[Ca^{2+}]_i$ in monolayers of cultured porcine coronary artery smooth muscle cells grown in 96 well plates. Responses are shown as mean (± SEM, n=4) increases in $[Ca^{2+}]_i$ in control cells (o-o), and in cells preincubated for 3 minutes with 0.1nM (●-●), 1nM (▽ - ▽) and 10nM (▼-▼) BQ-123. Inhibition is achieved at the 10nM concentration of antagonist only, significance being indicated as * ($P<0.05$), using ANOVA with *post hoc* Dunnett's test for comparison with controls.

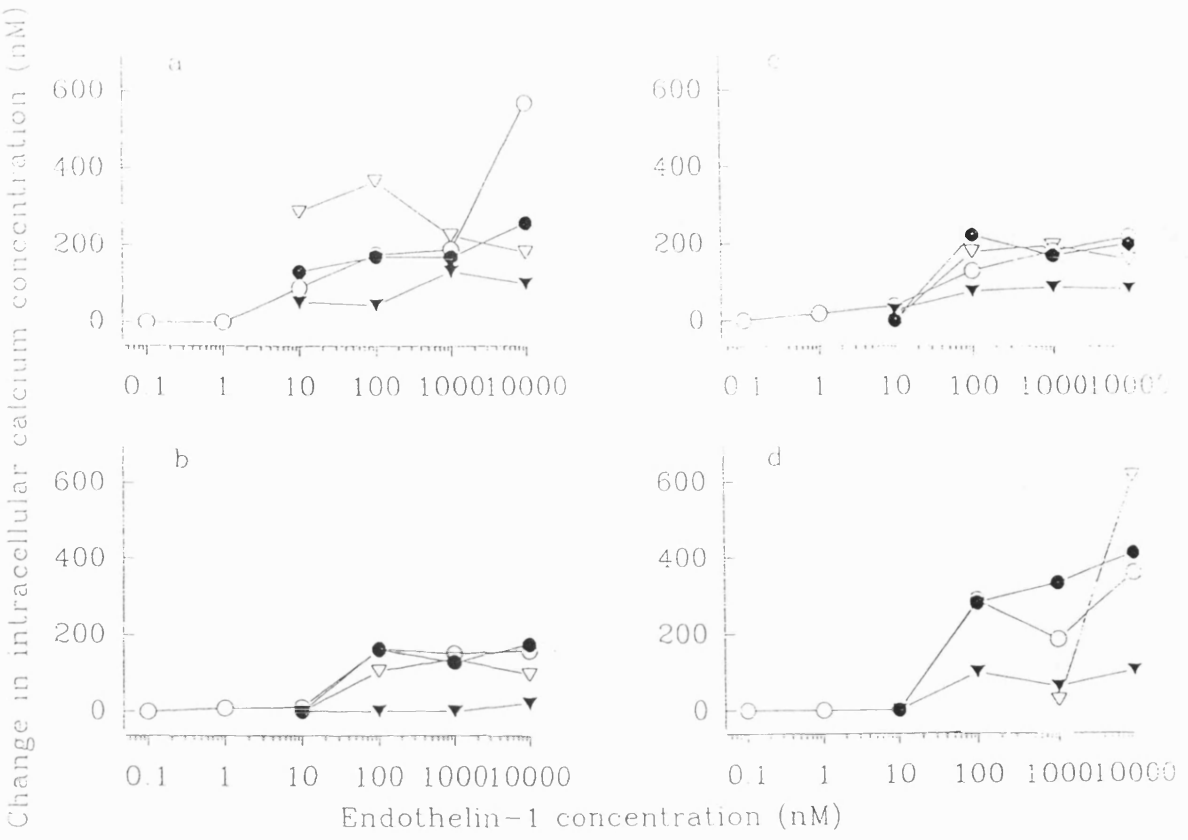


Figure R-23
Effects of BQ-123 on endothelin-1-induced increases in $[Ca^{2+}]_i$ in four separate experiments (a, b, c, and d). Controls (o-o) are compared with BQ-123 0.1nM (●-●), 1nM (▽ ▽), and 10nM (▼ ▼) respectively.

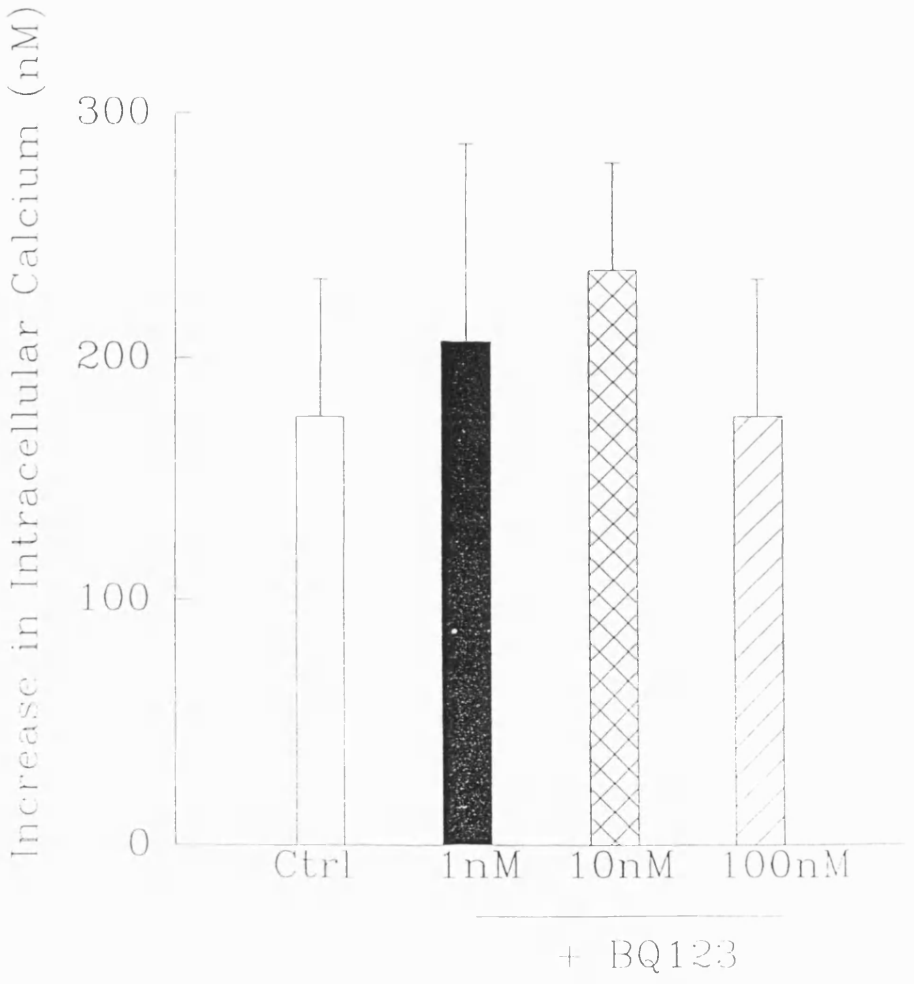


Figure R-24
Effects of BQ-123 (1-100nM) on $[Ca^{2+}]_i$ responses to bradykinin (1nM) in porcine coronary smooth muscle cell monolayers. Cells were preincubated for 3 minutes with BQ-123. Responses shown as mean ± SEM, n=4. No significant differences between means are seen on comparison using ANOVA.

6.1.3 Role of extracellular calcium in the endothelin-1 response

At the higher concentrations used, endothelin-1 elicited an initial peak response followed by a plateau phase of calcium elevation in most cell groups. The larger divalent ion, Ni^{2+} ($NiCl_2$, 5mM) was used to inhibit the influx of extracellular calcium by blocking calcium channels (e.g. Tsien *et al.*, 1988; James *et al.*, 1993), and its effects on both peak and plateau phases of the endothelin-1 response are seen in figure R-25. The differences between the responses are not statistically significant, but demonstrate an inhibitory trend in both the peak and plateau phases of the response.

6.1.4 Effect of sarafotoxin 6c on intracellular calcium

As with endothelins, the effect of sarafotoxin 6c on monolayers of smooth muscle cells was also variable. In two experiments, sarafotoxin 6c in concentrations of up to 10μM elicited no change in $[Ca^{2+}]_i$ when added to monolayers of smooth muscle cells (data not shown). However, a 5μM concentration elicited a ~180nM $[Ca^{2+}]_i$ increase in a separate preparation (R-26), subsequent addition of endothelin-1 (1μM) eliciting a further and larger calcium rise (peak change above basal of ~300nM).

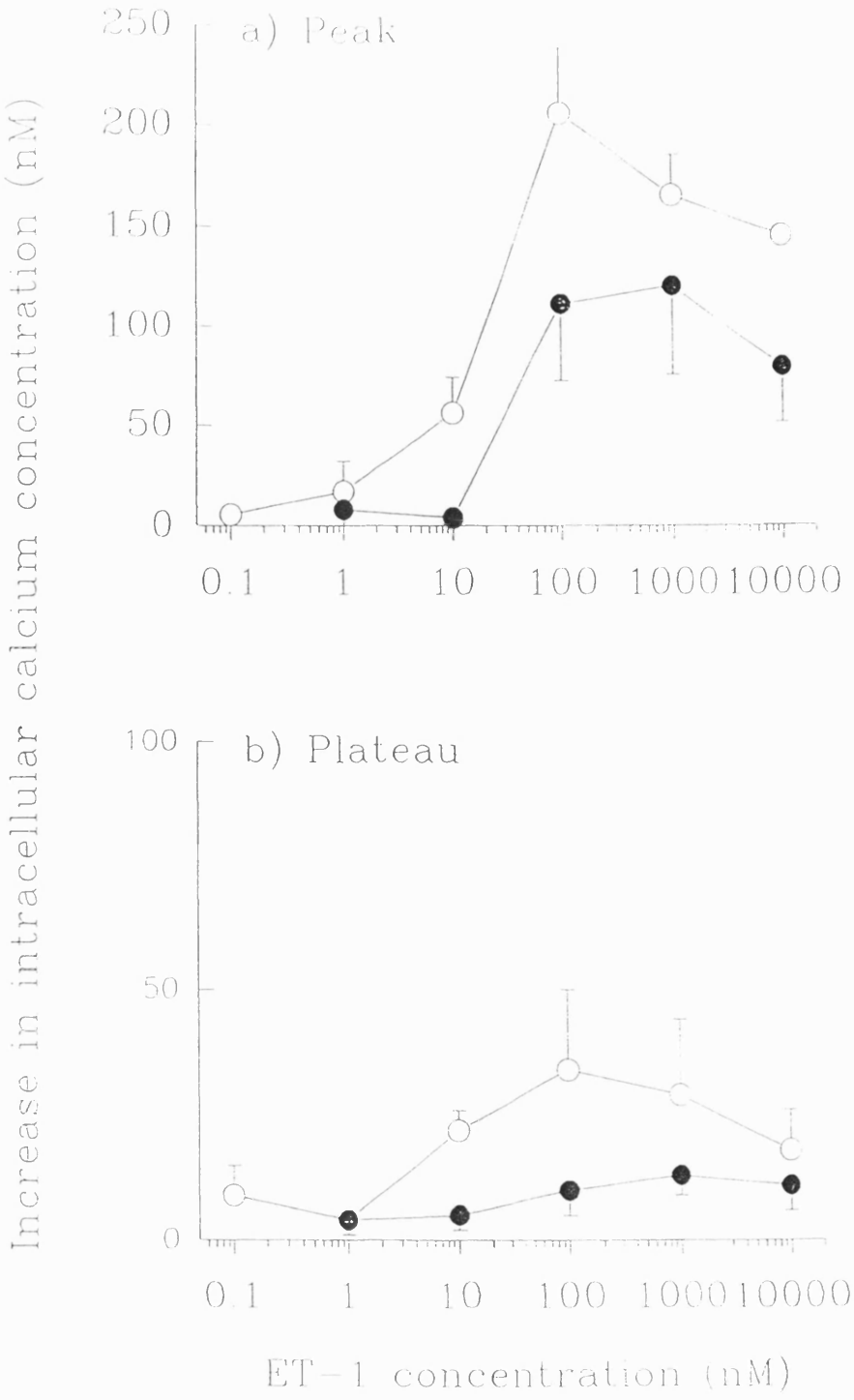


Figure R-25
The results of experiments investigating the role of extracellular calcium in the rise in $[Ca^{2+}]_i$ in response to endothelin-1 in porcine coronary smooth muscle cell monolayers. Mean (± SEM, n=4) increase in $[Ca^{2+}]_i$ in 1mM extracellular calcium (o-o) is compared with the responses elicited in the presence of 5mM extracellular $NiCl_2$ (●-●) in both the peak and the plateau phases. No significant differences are detected between means using Student's t-test.

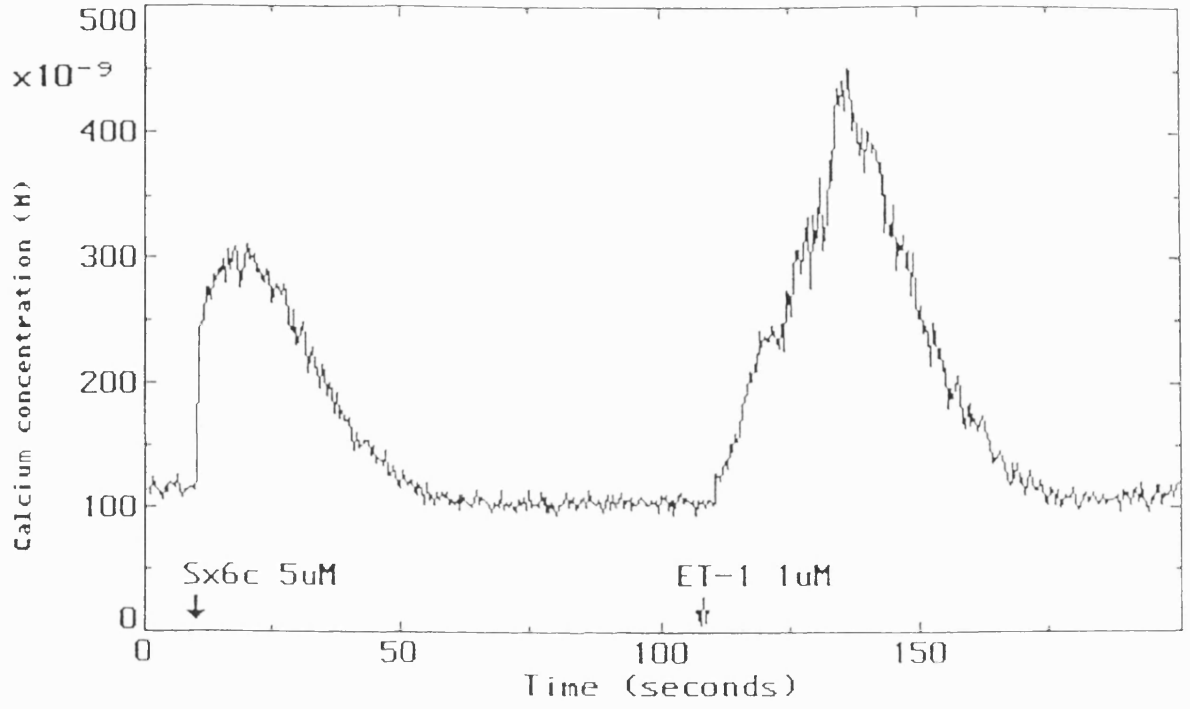


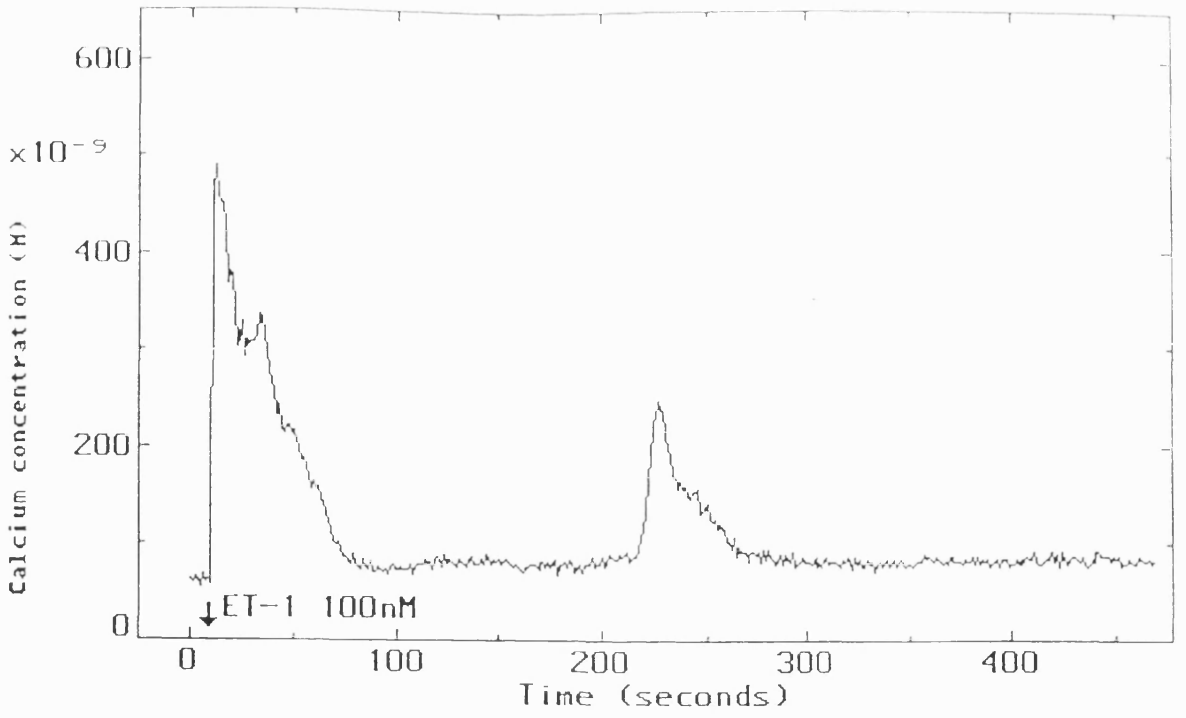
Figure R-26
The effect of sarafotoxin 6c and endothelin-1 added successively to a monolayer of porcine coronary smooth muscle cells. The initial increase in $[Ca^{2-}]_i$ to sarafotoxin 6c (5μM) returned to basal levels before addition of endothelin-1 (in the continuing presence of sarafotoxin 6c) initiated a further increase in $[Ca^{2+}]_i$.

6.2 Calcium responses in cells grown on coverslips

The measurement of calcium responses in single cells was not practicable with the magnification (20x objective) used for multi-well cell culture plates, hence the culture of cells on thin glass coverslips which allowed viewing of lower cell numbers and single cells using a 40x objective. These experiments were carried out at 37°C which was not possible in the multi-well plates. The basal $[Ca^{2+}]_i$ (73 ± 13nM, mean ± sem, n=4) in single cells measured under these conditions was similar (despite any temperature difference) to that in larger areas of cells in the 96 well plates when using ANOVA for comparison with monolayers and suspensions. However, it became apparent that agonist responses measured using small cell numbers, were highly variable, both in initial peak of the response to endothelin-1 and in the latency to response. For this reason, the remainder of section 6.2 concentrates on responses in single cells. None of the cells tested gave a response to vehicle alone (n=4).

6.2.1 Responses to endothelin-1 in single cells

It can be seen from figure R-27 that the addition of endothelin-1 to the chamber resulted in an initial peak increase in $[Ca^{2+}]_i$ with some variation in latency. This was followed by slow oscillations in $[Ca^{2+}]_i$ rather than by a plateau phase. The frequency of the oscillations was variable and not related to the endothelin concentration. This can be seen more clearly in figure R-28a, where the amplitude of the peak response as well as the oscillation frequency is different for two cells given the same endothelin concentration. The inconsistency in peak response between cells from the same preparation is highlighted in figure R-28b, where the initial component of the response to 100nM endothelin-1 is seen in four separate cells.



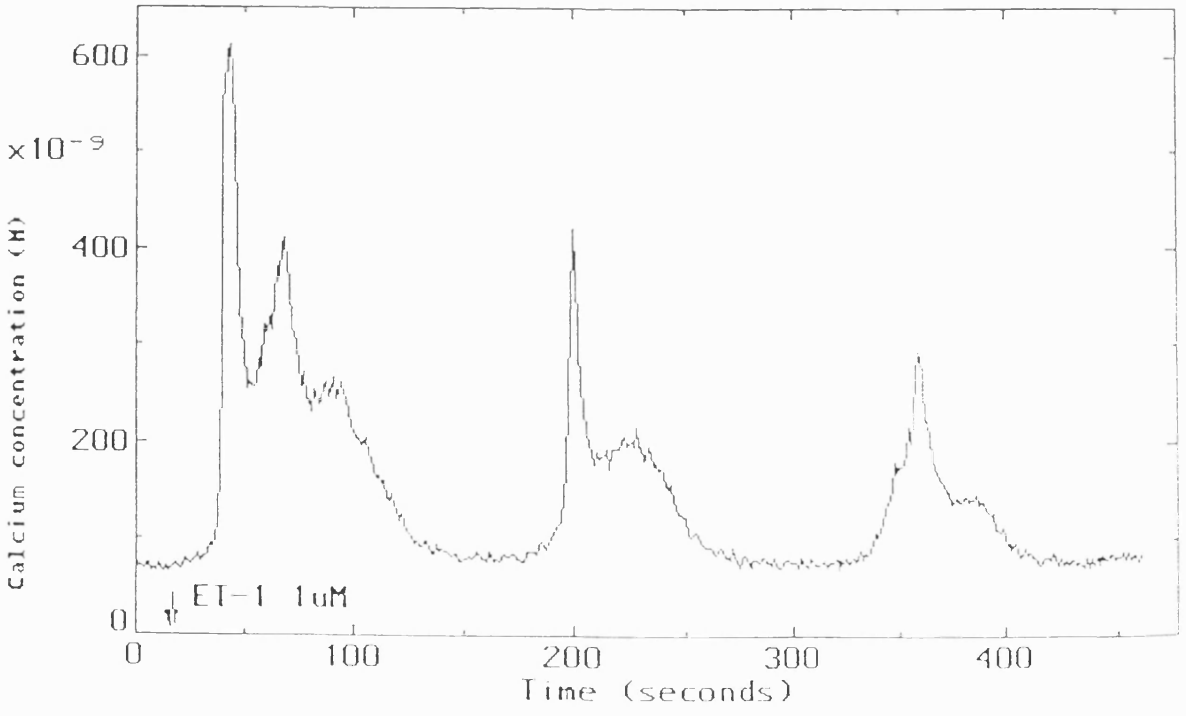


Figure R-27

The above traces show $[Ca^{2+}]_i$ in two single porcine coronary smooth muscle cells grown on glass coverslips. The time course and amplitude of responses to different concentrations of endothelin-1 added as indicated, are shown (100nM in upper panel, 1μM in lower panel).

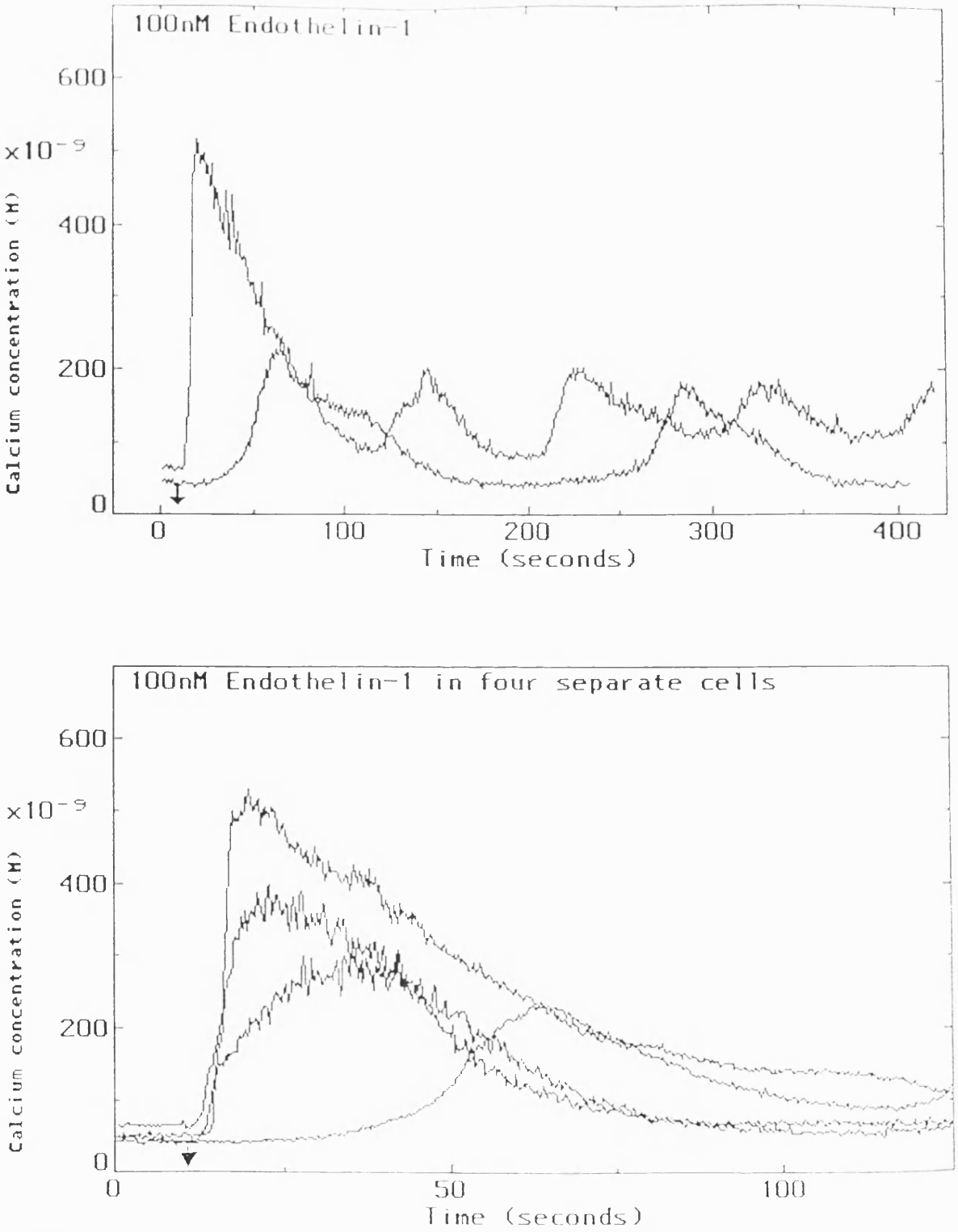


Figure R-28

Responses in $[Ca^{2+}]_i$ to a single concentration of endothelin-1 (100nM added as indicated) in single porcine coronary smooth muscle cells grown on coverslips. The time course of responses in two separate cells can be seen in the upper panel, highlighting the difference in frequency of the oscillatory response possible between cells. In the lower panel, the differences in the amplitude and latency of the initial peak $[Ca^{2+}]_i$ can be seen in four separate cells.

The variability of the $[Ca^{2+}]_i$ change was not confined to endothelin-1; oscillatory responses were also seen with 1μM bradykinin, with as much inconsistency in peak response as with endothelin-1 (not shown). In addition, of cells from four separate preparations, some individual cells (4 of 24) were unresponsive to 100nM or 1μM endothelin-1 whereas subsequent addition of 1μM bradykinin continued to elicit a clear response (not shown). In other cells responding to endothelin-1 (7 of 24), further response to bradykinin (1μM) was absent or reduced. 6 of 24 cells did not respond to either bradykinin or endothelin.
Owing to the marked variability of the initial calcium response and the number of preparations not responding to endothelin-1, the effect of BQ-123 was not determined.

6.2.2 Responses to sarafotoxin 6c in single cells

The responses of individual cells in different preparations to sarafotoxin 6c were also inconsistent. In three preparations, the maximum response to Sx6c was a 30nM increase in intracellular calcium above the basal level, which was not dose-related and could be described as "all or none". For comparison, a 100nM dose of sarafotoxin 6c produced no response in 2 preparations, a 29nM increase in a third cell and a 255nM increase above basal $[Ca^{2+}]_i$ with subsequent oscillations in another cell. This could indicate a sub-population of cells which are responsive to this ET_B receptor agonist. The cells not responding to sarafotoxin 6c all subsequently responded to a 100nM concentration of endothelin-1 (figure R-29).

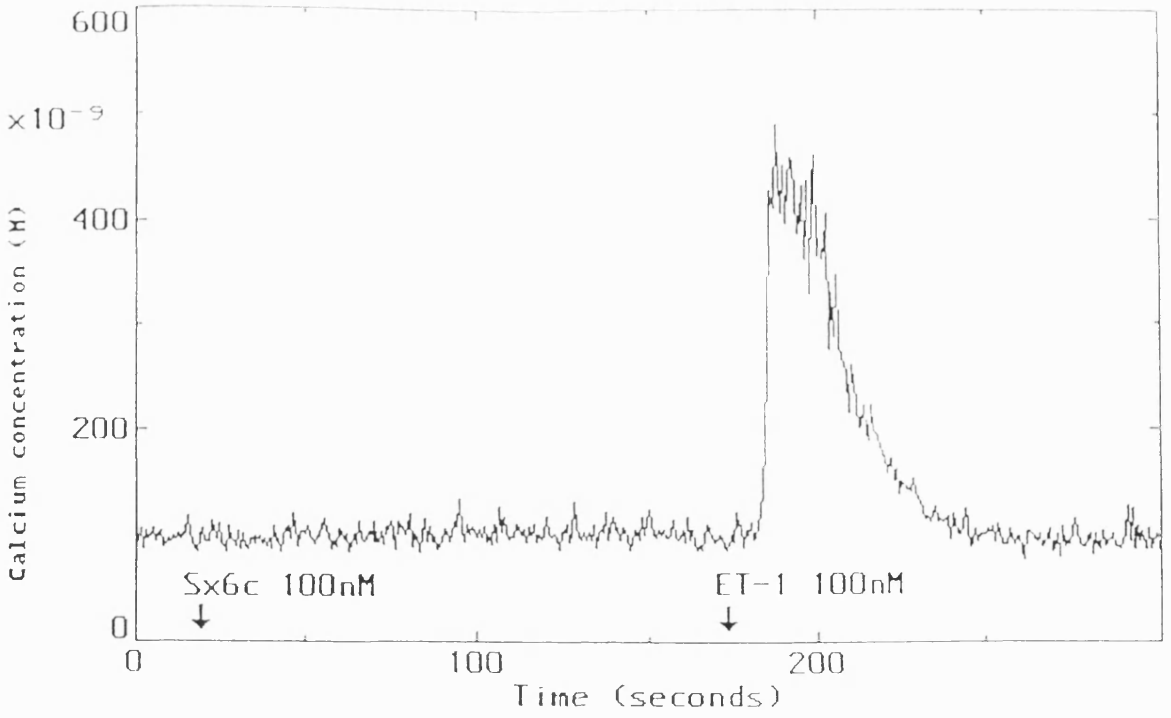


Figure R-29
The trace above illustrates the increase in $[Ca^{2+}]_i$ in response to endothelin-1 in a single cell, where no response was elicited following prior addition of sarafotoxin 6c (added as indicated by arrows).

6.3 Calcium responses to endothelin-1 in cell suspensions

To gain an overall view of the effect of endothelin-1 on large numbers of cells and hence the meaned effect on a population, the cells were grown in larger populations and dispersed. The mean basal intracellular calcium levels recorded in these preparations (calibrated using the method of Grinkiewicz *et al.*, 1985) were 226 ± 10nM (n=4), a level significantly higher than that measured in single cells (73 ± 13nM, n=4) or monolayers (73 ± 6nM, n=4; $P<0.001$ using ANOVA, and Tukey's test *post hoc*).

6.3.1 Intracellular calcium responses to endothelin-1 in populations of dispersed coronary smooth muscle cells

Endothelin-1 application resulted in a peak and a more consistent plateau phase of the $[Ca^{2+}]_i$ response in cell suspensions when compared with the fewer cell numbers in monolayers. The peak response to concentrations 3-300nM endothelin-1 is illustrated in figure R-30a, which shows a clear concentration relationship in the experiment illustrated. The endothelin-1-induced plateau phase was not concentration-related in its magnitude nor affected by any of the antagonist concentrations used in cell population experiments. The level of the plateau phase of calcium increase measured at 200s after agonist addition, was 40-85nM above the initial $[Ca^{2+}]_i$. There was no significant difference in the level of the plateau phase between the concentrations of endothelin-1 used (data not shown).

6.3.2 Effect of BQ-123 on responses to endothelin-1 in populations of dispersed coronary smooth muscle cells

As the inhibitory effect of 10nM BQ-123 in cell monolayers appeared to be non-competitive, the effect of this antagonist added simultaneously and with three minutes' incubation was compared as the non-competitive action of this antagonist has been linked to its duration of interaction with the agonist and receptor (Vigne *et al.*, 1993).

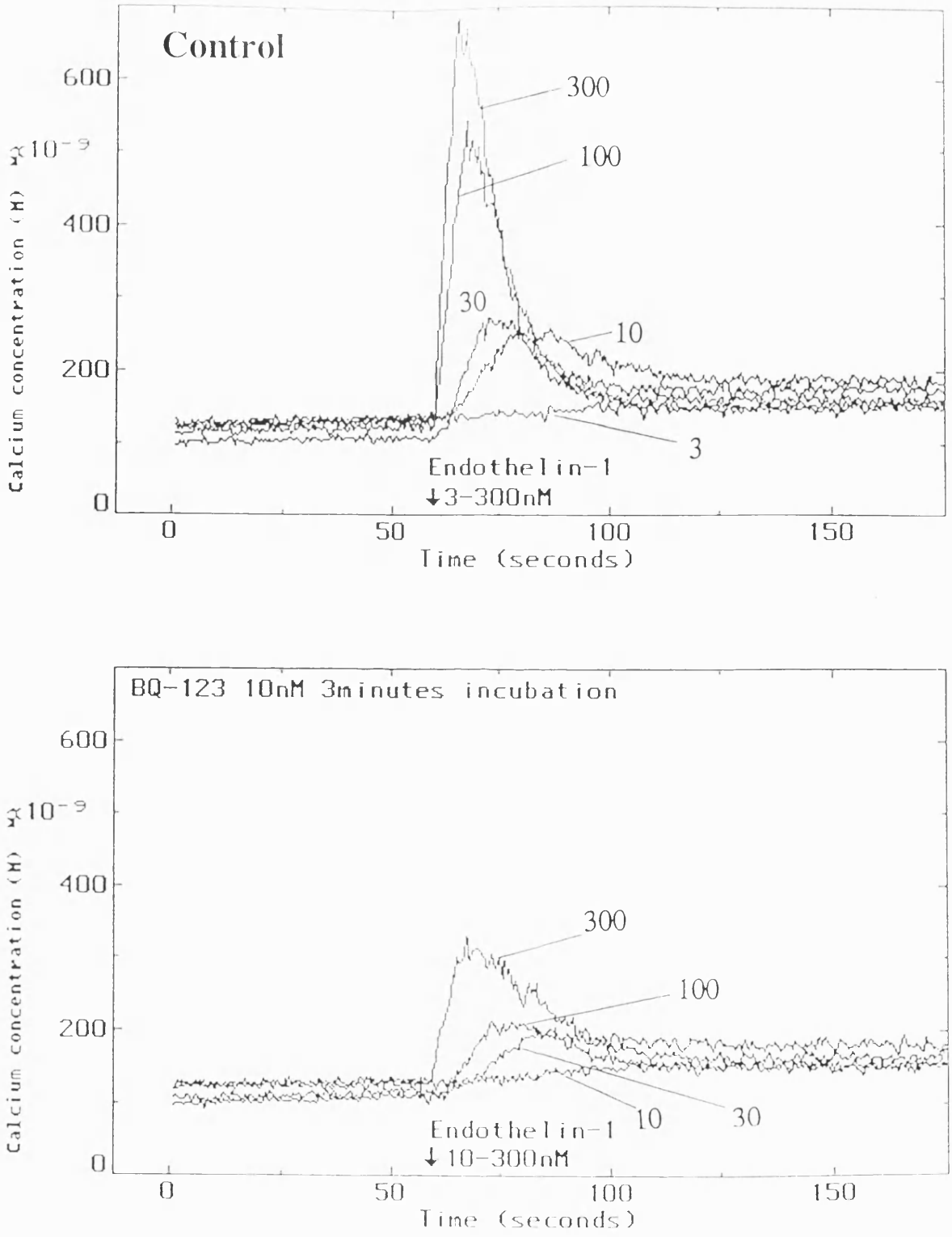


Figure R-30a & R-30b

The representative experiments above show the effect on $[Ca^{2+}]_i$ of concentrations of endothelin-1 added as indicated, to a suspension of porcine coronary smooth muscle cells. The upper panel (a) shows responses in control preparations, and the lower panel (b) the effects of 3 minutes' preincubation with 10nM BQ-123. Peak increase in $[Ca^{2+}]_i$ appears to be inhibited in this example by the ET_A receptor antagonist.

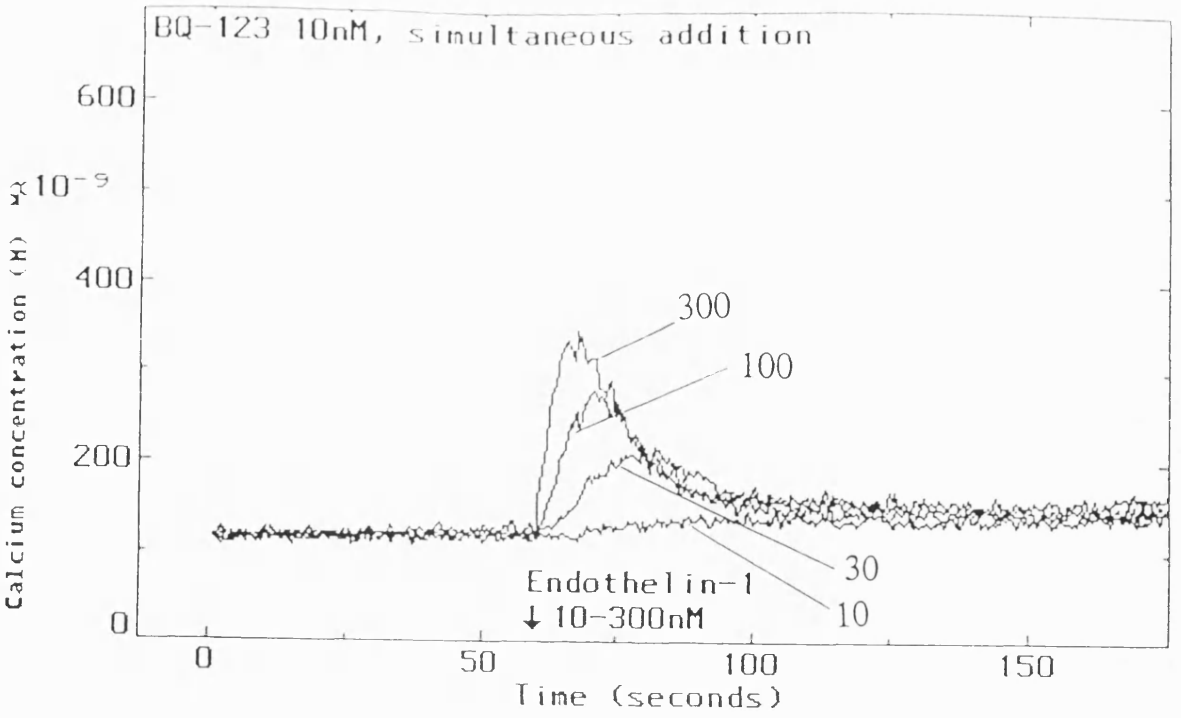


Figure R-30c
Responses in the same experiment where addition of BQ-123 (10nM) was simultaneous with the endothelin-1. The apparent inhibitory effect on responses, compared with R-30a, remains despite lack of equilibration time in this example.

The responses to endothelin-1 in the presence of BQ-123 remain concentration related, as seen in the traces in figure R-30b and R-30c. These demonstrate the effects of preincubation of cells with (R-30b) or simultaneous addition (R-30c) of BQ-123 (10nM). Both preincubation with the antagonist and its simultaneous addition appear to result in inhibition of the peak responses when compared with controls (R-30a).

The results of 4 experiments with control responses and where BQ-123 (3nM and 10nM) was preincubated with the cells for 3 minutes, are summarised in figure R-31. There are no significant differences from control values at any of the data points when compared by ANOVA.

The effect of simultaneous addition of BQ-123 (10nM) with the endothelin-1 is shown in figure R-32. Again, there is no significant difference from controls or from the data from preincubated cells.

From the data obtained, it is not possible to determine if the interaction with the 10nM BQ-123 is competitive or non-competitive.

Sarafotoxin 6c (100nM) was added in two experiments only but with no effect on $[Ca^{2+}]_i$, although cells subsequently responded to endothelin-1 (100nM).

6.3.3 Effect of herbimicin-A

Incubation of the cultured cells for 24 hours with herbimycin-A (875nM) caused no significant inhibition of the initial peak increase in $[Ca^{2+}]_i$ provoked by endothelin-1 in the cell suspensions. The combined results can be seen in figure R-33 (upper figure). There does, however, appear to be a trend toward inhibition of the peak response. There was no significant difference in the plateau phase of the response to endothelin-1 in herbimycin-A treated cells (R-33, lower figure).

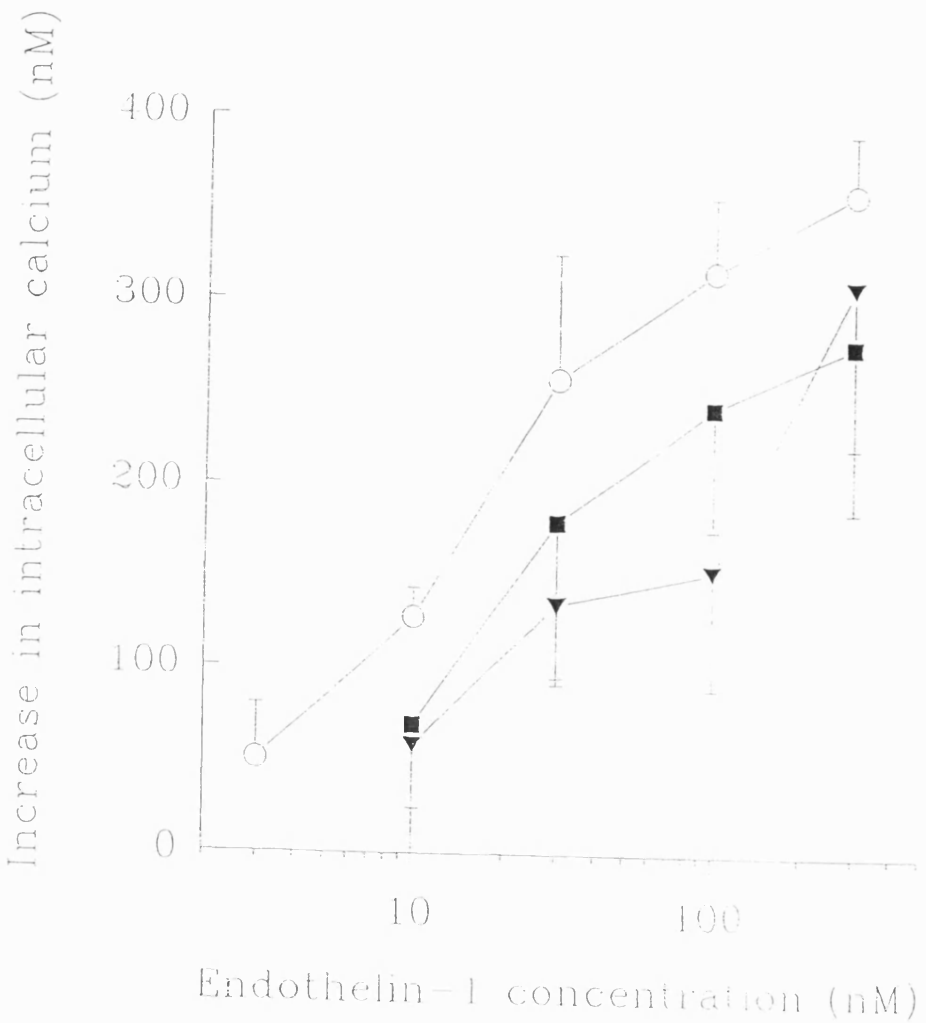


Figure R-31

The increase in $[Ca^{2+}]_i$ in response to endothelin-1 using large numbers of porcine coronary artery smooth muscle cells in suspension is shown as mean ± SEM (n=4). Responses in controls (o-o) are compared with those in cells from the same preparations preincubated with BQ-123 3nM (■ ■) and 10nM (▼ ▼) for 3 minutes. There are no significant differences between the mean responses (ANOVA).

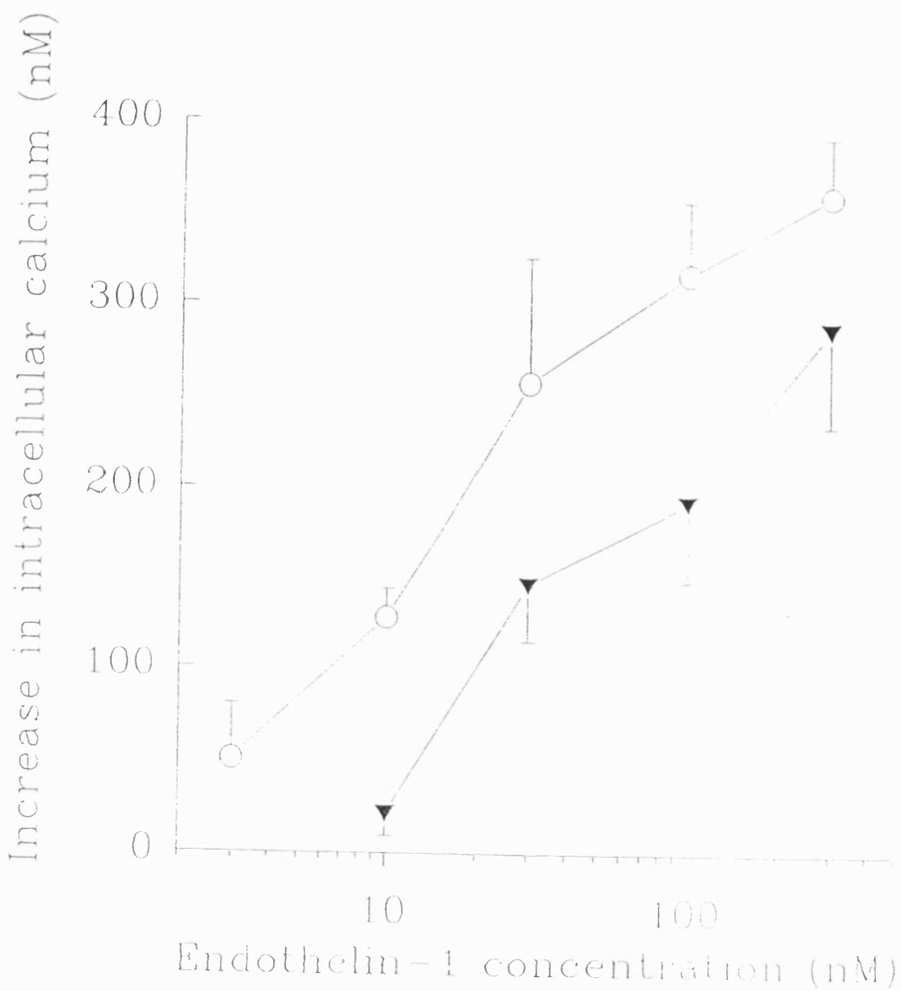


Figure R-32
The increase in $[Ca^{2+}]_i$ in response to concentrations of endothelin-1 in the same experiments as described in figure R-31 are shown as mean ± SEM (n=4). Controls (o-o) are compared with preparations where BQ-123 (10nM,) was added simultaneously with the endothelin (▾ ▾). There are no significant differences between means (ANOVA).

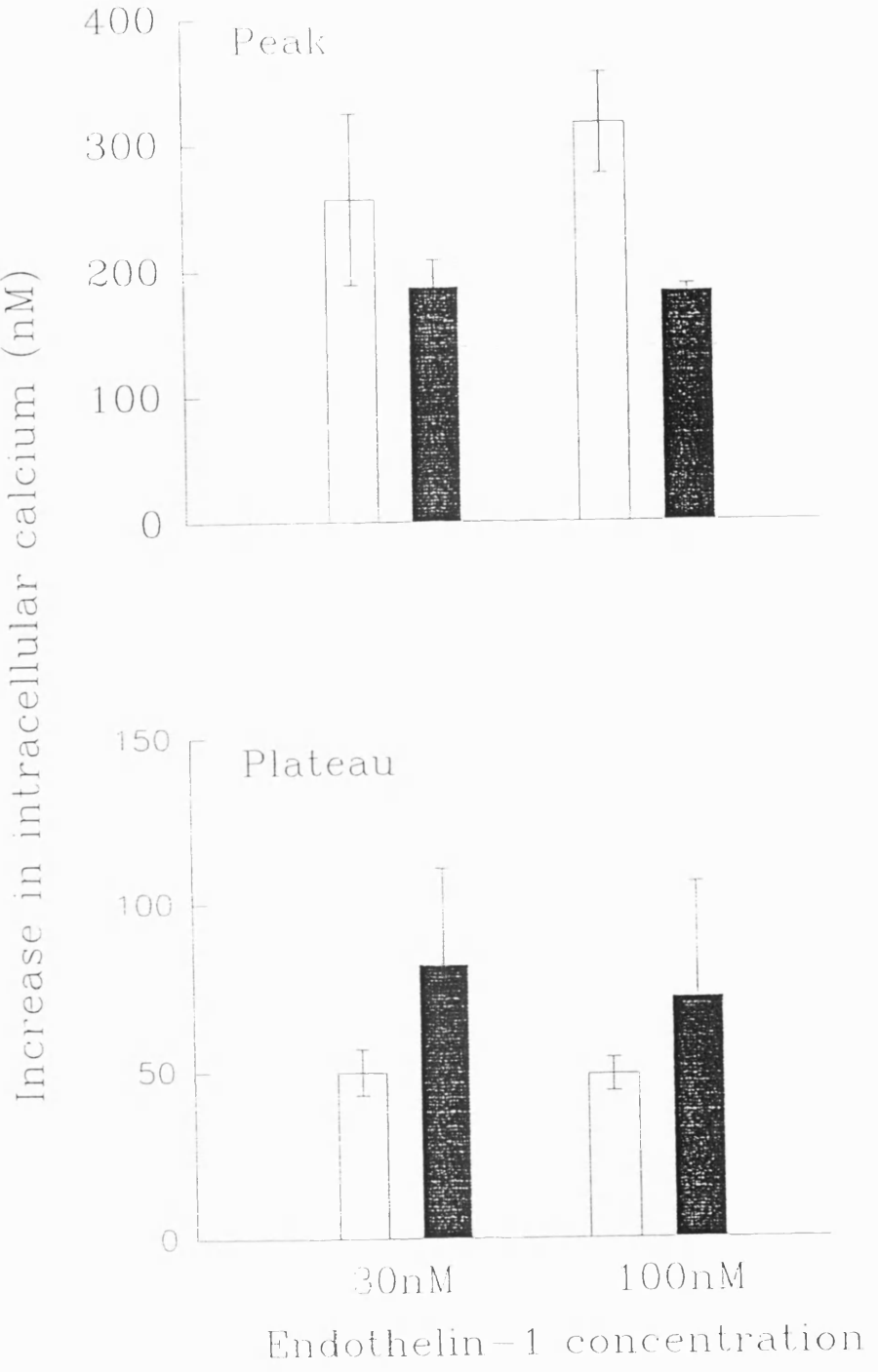


Figure R-33
The mean (± SEM, n=4) increases in $[Ca^{2+}]_i$ in porcine coronary smooth muscle cell suspensions in response to 30 and 100nM endothelin-1 are compared in control preparations (taken from the experiments shown in R-31 & R-32; open bars), and in preparations treated with herbimycin-A (875nM; filled bars) . The upper figure shows changes in peak initial response, while the lower figure gives data from the plateau phase of the responses. There are no significant differences when compared using ANOVA

DISCUSSION

7.0 Effects of ischaemia and reperfusion

The effect of ischaemia / reperfusion alone on coronary perfusion pressure, during the time course of the experimental protocol (figure R-1), was a steady rise. Although there is no significant difference when each point is compared with the control perfusion pressure, the rate of rise may be important in the interpretation of results measured cumulatively over the same period. The difference between mean perfusion pressure at the start of measurements and mean perfusion pressure at the end of the experimental period in controls was 15mmHg whereas the difference in ischaemic / reperfused hearts was 28mmHg. This is a large enough difference between means over the measurement time, to be considered when comparing a cumulative increase in perfusion pressure in ischaemic / reperfused hearts with control values.

The initial perfusion pressure is lower in post-ischaemic hearts which contributes to the difference in means at the start and end of the dose-response curve period. The mechanisms underlying this and the subsequent increase in perfusion pressure have not been explored here. However, the phenomenon of post-reperfusion vasoconstriction has been described in dog heart *in vivo* (Sobey *et al.*, 1990). The loss of release of endothelially produced mediators of vasodilatation has been cited as a contributory cause (Sobey & Woodman, 1993), damage to, or stunning of the endothelium of microvessels being a sequel of ischaemia (Nevalainen *et al.*, 1986; Kim *et al.*, 1992). It is possible also that the increased release of vasoconstrictor substances during ischaemia or after reperfusion may contribute to this phenomenon. These may include endothelin-1 itself which has been shown to be released from rat hearts after reperfusion (Brunner *et al.*, 1992), and endothelium-derived lipoxygenase products as well as noradrenaline and 5-hydroxytryptamine (Kwan *et al.*, 1990). The presence of increased concentrations of endothelin-1 could be important in potentiation of the effects of both 5-hydroxytryptamine and noradrenaline (Yang *et al.*, 1990; Nakayama *et al.*, 1991) and this would exacerbate the rise in perfusion pressure seen. Whatever the underlying mechanism, the increase in basal perfusion pressure makes interpretation of cumulative vasoconstriction problematic.

7.1 Effects of ischaemia and reperfusion on endothelin and sarafotoxin 6c responses:

Binding sites for α_1-adrenoceptor agonists have been shown to increase after a 30 minute period of ischaemia (Allely & Brown, 1988) in rat hearts *in vivo* but these changes were not confirmed by a later study *in vitro* (Dillon *et al.*, 1988) and do not necessarily represent an increase in functional receptors. The experiments on the effects of endothelins after ischaemia / reperfusion were performed in an attempt to discover whether the increase in endothelin binding sites described by Liu *et al.*, (1990) represented an increase in functional receptors, following a similar protocol *in vitro* to that which revealed the increased binding sites.

The change which was seen in the current study in the responses to endothelins after ischaemia / reperfusion can be separated into the morphological change in the responses and the apparent increase in the magnitude of the responses.

7.1.1 Morphological changes

The morphology of the endothelin-1 and -2 responses after ischaemia / reperfusion appeared to change at doses of each isopeptide where a vasodilator component was present in control hearts (see figure R-4). The ischaemic / reperfused preparations showed a loss of the vasodilator component accompanied by a change in the morphology of the upstroke of the vasoconstriction which appeared more acute. The responses to endothelin-3 where vasoconstriction occurred in control hearts were not in a range at which vasodilatation was a feature and so the morphological change is less apparent. The changes identified in this study show similarities to changes in the responses to endothelin-1 described after injection of air through the coronary vasculature to remove endothelium (Baydoun *et al.*, 1989). Following air injection, the response to a single dose of endothelin-1 lacks any vasodilator component and appears to be more acute in onset than that seen in the control preparations (see figure D-1) as well as being of greater magnitude than the control.

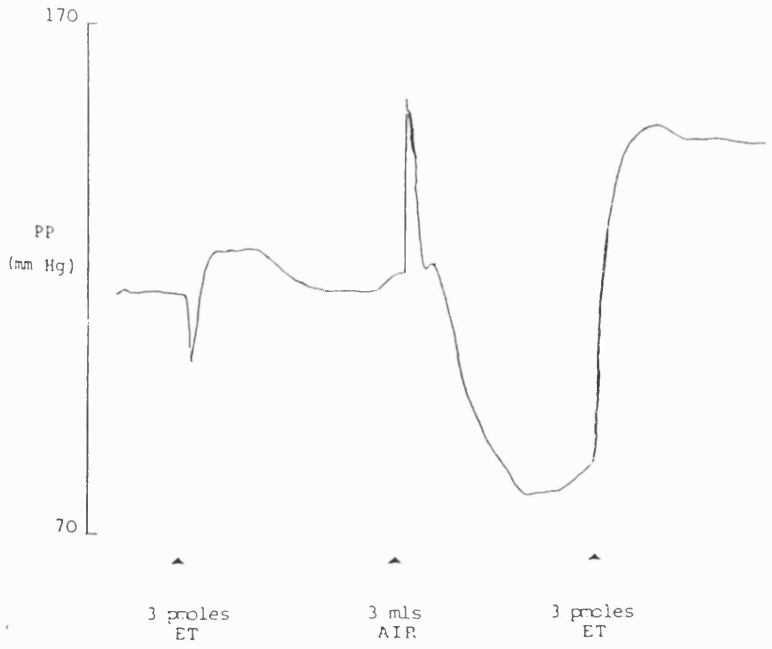


Figure D-1
The effect of injection of air through the coronary circulation of the rat isolated heart on the perfusion pressure response to a bolus dose of endothelin-1 (3pmol). (Baydoun *et al.*, 1989).

7.1.2 Changes in magnitude

Changes in the magnitude of the responses to all three endothelins are more difficult to differentiate. At first approximation, the results suggest that responses to all three endothelins are enhanced after ischaemia / reperfusion (figures R-7 & R-8). However, it is probable that at least part of this enhancement is due to the inclusion of a rising basal perfusion pressure in the cumulative measurement, and separation of this component is not possible. It is likely that part of any real increase would be contributed by loss of a functionally opposing vasodilatation, but to what extent this is important is not clear.

The experiments of Neubauer *et al.*, (1991) describe an enhancement of endothelin-1-induced reduction of coronary flow after 30 minutes of ischaemia followed by reperfusion. Determination of coronary resistance by measurement of flow (see introduction section 1.8.1) would lead to a similar inclusion of any increase in basal coronary resistance similar to that seen in the current study. The effects of ischaemia and reperfusion alone on

coronary flow were not described by Neubauer *et al.*, (1991), and it is conceivable that a reactionary vasoconstriction would be manifest over the same period in their experiments as was demonstrated here by an increase in perfusion pressure.

McMurdo *et al.*, (1991) show an enhancement after ischaemia / reperfusion, not of magnitude of the response to a single 100pmol dose of endothelin-1, but of its duration. Use of a single bolus dose removes much of the problem arising from cumulative measurement of vasoconstrictor effect on perfusion pressure. The authors of this study suggest that the prolongation may be due to impairment of prostacyclin production which would otherwise oppose the vasoconstriction, as indomethacin further potentiated the duration of response. However, this study demonstrated no vasodilator component to the endothelin-1 response in control hearts, despite use of a model similar to that in the current study.

The only conclusion that can be drawn from these results, in the light of the rise in basal perfusion pressure, is that the attenuation of the dilator phase of the response after ischaemia / reperfusion is common to all three endothelins and to sarafotoxin 6c, and this loss could contribute to vasoconstrictor enhancement as described by Neubauer *et al.*, (1991).

7.2 Selectivity of the changes in the endothelin response: vasoconstrictors and vasodilators

The selectivity of the changes in the responses of the endothelins and sarafotoxin 6c was tested by use of the same protocol for other coronary constrictor and dilator substances. The coronary vasodilatation produced by all the agents used except verapamil was significantly attenuated after 30 minutes of ischaemia followed by reperfusion. This suggests that attenuation of vasodilatation is common to agents acting by different mechanisms, both endothelium-dependent and independent. Impairment of coronary relaxation after ischaemia / reperfusion is a well documented event, having been

demonstrated in different preparations after varying durations of ischaemia and reperfusion. Most of the data produced to date has been indicative of an impairment of endothelial function; for example the responses to bradykinin and acetylcholine, but not sodium nitroprusside, have been shown to be reduced after ischaemia / reperfusion in dogs (Mehta 1989; Kim *et al.*, 1991) and in porcine vessels (Dignan *et al.*, 1992). Responses to thrombin (Ku, 1982) have also been attenuated after an ischaemia / reperfusion protocol in dogs. The phenomenon of "endothelial stunning" has been cited as a result of brief ischaemia, the temporary effect inducing loss of vasodilatation (Kim *et al.*, 1992). However, using the current protocol, responses to some agents acting directly on vascular smooth muscle (adenosine, sodium nitroprusside and papaverine) have also been attenuated. In the case of vasodilator agents which may not be dependent on the endothelium for their action, there are also previous examples of loss of responsiveness in different preparations. Attenuation of the adenosine response in the dog has been ascribed to the effects of ischaemia / reperfusion (60 minutes of each) on a specific (A_2) receptor (Cox *et al.*, 1994) and the reduction of adenosine and papaverine responses to "microvascular stunning" (Triana & Bolli, 1991), after a brief period of ischaemia (15 minutes) and persisting for 4 hours after reperfusion. "Microvascular stunning", whatever the mechanism(s), is clearly not confined to endothelium-dependent responses and appears also to be a feature of the protocol used in the current study. Vasodilatation in response to verapamil is as a result of an effect on L-type calcium channels, a response which does not require an intracellular signal transduction component or the utilisation of high energy phosphates. It could therefore be argued that vasodilator responses to agents requiring receptor activation or intracellular transduction processes will be more greatly attenuated than responses to these vasodilators. Further work would be required to elicit the reasons why signal transduction of vasoconstriction responses does not appear to be similarly affected if this is the case.

There was no enhancement of the vasoconstriction produced by either phenylephrine or Bay K 8644, the response to neither of which has a vasodilator component. As these responses returned to baseline values rapidly they were not measured cumulatively, so there can be no contribution to the vasoconstriction from a baseline rise in perfusion pressure, which is a

problem with the endothelins. This lack of significant effect of a period of ischaemia with subsequent reperfusion on responses to phenylephrine concurs with the results of a study by McMurdo *et al.* (1991), where the ischaemia / reperfusion protocol is similar. The enhancement of the duration of vasoconstriction to a single bolus dose of endothelin-1 was not found with either phenylephrine or U46619 (a thromboxane analogue).

It was necessary to test the effects of this particular protocol, including a period of 30 minutes of ischaemia and 10 minutes of reperfusion, on responses to other vasoconstrictors as previous studies have shown contradictory results. For example, following 15 minutes of ischaemia in dogs, vasoconstrictor responses to U46619 and potassium were enhanced (Kim *et al.*, 1992). This concurs with the results of an earlier study in dogs in which both potassium and ergonovine responses were enhanced after 60 minutes each of coronary occlusion and reperfusion (Van Benthuysen *et al.*, 1987). A separate study describes the progressive attenuation, rather than enhancement, of a vasoconstrictor response to potassium in isolated porcine coronary vessels which were removed following increasing periods of ischaemia *in vivo* (Dignan *et al.*, 1992). Hence the effects of ischaemia and reperfusion on responses to vasoconstrictor substances are variable and may be dependent on the period of ischaemia involved and possibly on the species or preparation used.

7.3 Mechanism of the vasodilator component of the endothelin-3 response

No information on the mechanism of the vasodilatation induced by the endothelins in this preparation could be gleaned from the results of the ischaemia / reperfusion experiments as the "microvascular stunning" phenomenon appears to be non-selective. Hence the isopeptide with the clearest vasodilator effect, endothelin-3, was selected for further study. The inhibition of the vasodilator component by use of an antagonist would create a situation where the effects of loss of the vasodilator component on the vasoconstriction phase could be assessed in the absence of ischaemia / reperfusion.

This was initially attempted by perfusing a combination of indomethacin and L-NMMA. However, the effect of this perfusate on the response to a submaximal vasodilator dose of endothelin-3 was not significant, despite a marked inhibition of thrombin- and bradykinin-induced vasodilatation. Therefore the inhibition was insufficient for an assessment of the effect of vasodilator attenuation to be made. No significant enhancement of vasoconstriction was apparent under these experimental conditions.

In the rat coronary circulation the evidence for the mechanism of vasodilatation by endothelin-3 is incomplete. There are reports of a role for prostacyclin in the vasodilator component of the endothelin response in hearts of other species, such as the guinea pig (Karwatowska-Propopczuk and Wennmalm, 1990) and dog (Okamura *et al.*, 1992). In porcine coronary artery, a mediator of vasodilatation has been identified as endothelially produced nitric oxide (NO), (Pernow & Modin, 1993; Ushio-Fukai *et al.*, 1992). However, there is little evidence for the release of NO in rat coronary artery, although other rat tissues have been shown to produce this substance in response to endothelins (Lawrence & Brain, 1992, in rat skin; Warner *et al.*, 1989, in rat mesentery). In the present study, there was no increase in basal perfusion pressure in response to perfusion of combined indomethacin (10μM) and L-NMMA (100μM). This suggests that the role of constitutively produced endothelial NO in the modulation of basal coronary artery tone in this preparation is not an important one. However, one report of basal coronary release of NO in the rat uses a higher L-NMMA concentration (500μM) than that used in the current study (Amrani *et al.*, 1992). A higher concentration of the NO synthase inhibitor might therefore have been used with better effect.

An inhibitor of ATP-sensitive potassium currents ($I_{K_{ATP}}$), glibenclamide, was also used in order to attempt inhibition of the vasodilator component of the endothelin-3 response. The vasodilator effects of endothelins in the vasculature have been attributed to a hyperpolarising effect which may be a result of direct action upon endothelin receptors in smooth muscle, or via release of endothelium-derived hyperpolarising factor (EDHF) both of which may result in opening of potassium channels. This potassium channel activation has been demonstrated in endothelin-induced vasodilatation of the cat pulmonary circulation

and this has been shown to be sensitive to the $I_{K_{ATP}}$ channel inhibitor glibenclamide (Lippton *et al.*, 1991). The involvement of an outward potassium current in the rat heart has also been demonstrated (Sakuma *et al.*, 1993), where an increase in the potassium concentration of the perfusate abolished the coronary dilator component of the endothelin-3 response.

In the current study, the perfused glibenclamide concentration was sufficient to inhibit the vasodilator response to an opener of $I_{K_{ATP}}$ channels, lemakalim, although the vasodilatation produced by endothelin-3 was significantly enhanced after glibenclamide perfusion, rather than inhibited (figure R-14a). This enhancement is likely to be an effect of the significantly higher basal perfusion pressure which was present in glibenclamide-perfused hearts. Hence no confirmation of the involvement of the $I_{K_{ATP}}$ channel in the vasodilator effect of endothelin-3 can be made from this study. This is supported by the results of another study in rat hearts where glibenclamide was ineffective in modifying the vasodilator component of the endothelin-3 response (Sakuma *et al.*, 1992).

However, vasoconstriction to endothelin-3 was attenuated after glibenclamide perfusion, this being a significant effect at 20, 100 and 200pmol doses ($P<0.05$; see figure R-14c). This is interesting in terms of the depolarising effect of endothelin acting via blockade of IK_{ATP} in porcine coronary smooth muscle reported by Miyoshi *et al.*, (1992). If this blockade were important in the vasoconstrictor response to endothelin-3 in rat coronary smooth muscle, then prior treatment with the antagonist glibenclamide, might be expected to limit the endothelin's depolarising effect, and hence vasoconstriction. This has in fact occurred, suggesting that one mechanism of the vasoconstrictor component of the response may be the inhibition of IK_{ATP}, although this cannot be the only mechanism as a substantial vasoconstrictor response remains. There may also be a contribution to the reduced vasoconstrictor activity from the increased basal perfusion pressure, where increased vasodilator activity obscures part of the vasoconstriction.

7.4 Effects of BQ-123 on vasoconstriction: receptor involvement in phases of the endothelin responses

The role of different receptors for endothelins in the different phases of the response was investigated using the ET_A-selective competitive antagonist BQ-123. Coronary constrictor responses to both endothelin-1 and endothelin-3 were significantly attenuated following perfusion with BQ-123 (figure R-15). This compound has been shown to be highly selective for the ET_A receptor; the IC_{50} value of BQ-123 against [^{125}I]endothelin-1 binding to vascular smooth muscle has been measured at 7.3nM, compared with 18000nM at cerebellar ET_B receptors (Ihara *et al.*, 1991), and similar figures have been obtained in separate studies (e.g. Sakamoto *et al.*, 1993). The reduction of the vasoconstrictor response to endothelins by BQ-123 therefore suggests that at least part of this constriction is mediated via ET_A receptors. This is also in keeping with the relative potencies of the endothelins in causing coronary constriction, the doses of endothelins -1 and -2 required to produce ~100mmHg increases in perfusion pressure being similar whereas (>10x) higher doses of endothelin-3 were necessary to produce a comparable level of effect (see figures R-7 & R-8). The ET_B-selective agonist sarafotoxin 6c was ineffective in producing vasoconstriction (figure R-8), which is consistent with there being no role for ET_{B2} receptors in endothelin-induced vasoconstriction.

The receptor subtype involved in the coronary constrictor response in various species has been the subject of investigation. An early study by Ihara *et al.*, (1991) investigated the binding of radiolabelled endothelin-1 and endothelin-3 to porcine coronary arteries and suggested that only ET_A receptors are found on the vascular smooth muscle. However in the same preparation the coronary constrictor phase of the response appears to be mediated by a receptor subtype which does not conform to the profile of either "known" receptor (Harrison *et al.*, 1992). In this species also, the ET_B (ET_{B2}) receptor has been implicated in this contraction, but this required the removal of the endothelial barrier (Shetty *et al.*, 1993) suggesting a site on the underlying smooth muscle. A further study (Schoeffter & Randriantsoa 1993) describes contraction mediated by both ET_A and ET_{B2} receptors on

porcine coronary smooth muscle, as well as a further component to the contractile response, which supports the results described by Harrison *et al.*, (1992). In man, Bax *et al.*, (1993) suggest a subtype of the ET_A receptor is present on coronary artery, as well as ET_{B2} which could be responsible for contractile responses, and Seo *et al.*, (1994) describe constriction mediated by both ET_A and ET_{B2} receptors in human coronary.

There is little data referring to an ET_{B2} mediated vasoconstriction in rat heart, but a study by Balwierczak (1993) suggests that both ET_A and ET_{B2} receptors are involved in vasoconstriction in this preparation, as an ET_B selective agonist (IRL 1620) induced this effect. The differences in the findings between this and the current study are difficult to reconcile, as the results here show no coronary constriction to the ET_B selective agonist sarafotoxin 6c (Williams *et al.*, 1991). However, the role of the endothelium in protection against ET_B mediated vasoconstrictor action in this preparation has not been clarified. The study performed by Balwierczak (1993) showed no vasodilator phase to the endothelin response and involved a higher ($20ml.min^{-1}$) flow rate. Though endothelial denudation was not performed, the higher flow rate involved might have been responsible for some increased drug access to underlying smooth muscle. However, confirmation of this would require investigation of the integrity of the endothelium under the experimental conditions in each study.

There was no attenuation of the coronary dilator component of the response to either endothelin-1 or endothelin-3 with BQ-123, suggesting that this phase is not ET_A receptor-mediated. In fact the duration of vasodilatation to endothelin-1 is prolonged (figure R-18), possibly because the later part of dilatation in controls is partly masked by the constrictor phase. The response to endothelin-3 was not prolonged, however, possibly because vasoconstriction is less marked in the dose range where the vasodilatation is present (10-100pmol). The vasodilatation response has been associated with ET_{B1} receptors found on endothelium (Sakurai *et al.*, 1990). This receptor is not selective for the different endothelin isopeptides and this is also consistent with the vasodilatation seen in these experiments in the isolated perfused rat heart, where all three endothelins and sarafotoxin 6c act as coronary dilators in the 10-100pmol dose range.(see figures R-5 & R-6).

The information gained in the current study is limited in terms of the nature of the endothelin receptors involved in both phases of the response. The subtype of receptor involved could be further investigated using a wider range of antagonists, including the ET_B-selective BQ-788 (Ishikawa *et al.*, 1994).

7.5 Desensitisation of the coronary dilator effect of endothelin-1

The experiments performed exploited the cross tachyphylaxis seen between the endothelin isopeptides which is extended to the ET_B selective agonist sarafotoxin 6c. The response to a single submaximal dose of endothelin was completely inhibited following a protocol of desensitisation using three 100pmol boluses of sarafotoxin 6c at 10 minute intervals. The lack of effect of this protocol on the responses to other coronary dilator and constrictor agents shows the selectivity of the desensitisation. This sarafotoxin 6c-induced tachyphylaxis is strongly indicative that the coronary dilatation produced by endothelins in the rat is mediated by the ET_{B1} receptor subtype.

Cross desensitisation has previously been demonstrated by Le Monnier de Gouville & Cavero (1991) in endothelin-induced hypotension in the rat, and is normally associated with the vasodilator phase of the endothelin response mediated by ET_{B1} receptors. In one report, however, a sarafotoxin 6c constrictor response has been the subject of tachyphylaxis (Sudjarwo *et al.*, 1993). The mechanism of desensitisation has been investigated by Hollenberg *et al.*, (1993) and has been shown to depend on the internalisation of endothelin-receptor complexes, as when this process was antagonised using dansylcadaverine, the desensitising effect was inhibited. In another study, the action of a C-terminal portion of endothelin-1 on the guinea pig ileum did not cause desensitisation, this segment of the peptide being unnecessary for the strong binding of the endothelin-receptor complex (Miasiro *et al.*, 1993). Hence the internalisation of tightly bound receptor complexes as a route for desensitisation is supported by their study. The method used in the current study, a maximal sarafotoxin 6c bolus repeated at 10 minute intervals, may be causing the

maximum degree of receptor binding and internalisation, and hence loss of vasodilator effect.

The complete loss of the coronary dilatation after desensitisation is accompanied in the current study by a comparable degree of vasoconstrictor enhancement. That the difference between vasodilator loss and vasoconstrictor enhancement is so small (a mean of 25mmHg vasodilator loss, compared with 28mmHg mean vasoconstrictor enhancement) would suggest that the enhancement could be a direct consequence of a loss of functional antagonism. This is an indication that vasodilator loss alone could be enough to result in an increase in vasoconstrictor effect similar to that seen after ischaemia and reperfusion.

8.0 Conclusions from experiments performed in the isolated perfused rat heart preparation

The responses to all three endothelins in the isolated rat heart consisted of a dilator and a constrictor phase in keeping with reports of previous authors (Baydoun *et al.*, 1989). Responses to the ET_B-selective agonist sarafotoxin 6c in the same preparation were almost completely vasodilator in character. The effect of ischaemia / reperfusion included a general rise in basal perfusion pressure which hindered interpretation of the cumulative measurement of vasoconstrictor responses in the reperfused hearts after a 30 minute period of ischaemia. The apparent increase in endothelin vasoconstrictor effect is therefore unhelpful and a better model is required. What can be stated with confidence, however, is that the coronary dilator component of the responses to endothelins -1, -2 and -3 as well as sarafotoxin 6c is markedly attenuated after the ischaemia / reperfusion protocol described in these experiments and this effect is common to all vasodilators tested except verapamil, where vasodilator loss was not significant.

Loss of the vasodilator component of the endothelin responses is in itself enough to result in an increase in vasoconstrictor effect as demonstrated where the coronary dilator component of a response to endothelin-1 was desensitised using a repeated dose of sarafotoxin 6c. The degree of enhancement of vasoconstriction was almost exactly equivalent to the level of vasodilatation lost, suggesting that a functional antagonism is important in the modulation of the action of the peptides. This could be of pathological importance where local endothelial disruption (such as in atheroma or after percutaneous transluminal coronary angioplasty) results in a loss of an endothelium-dependent vasodilatation to endothelin which has been shown in some preparations (Folta *et al.*, 1989). This could result in local vasospasm mediated by the endothelin peptides.

The mechanism of the coronary dilator component of the endothelin-3 response in the rat heart is not clear. From use of the agents L-NMMA and indomethacin it appears that NO synthase and cyclooxygenase products are not of primary importance in endothelin-induced vasodilatation. The inhibitory effects of the IK_{ATP} antagonist glibenclamide, suggest a

possible role for the closure of this ion channel in the constrictor phase of the response (in agreement with the results of Miyoshi *et al.*, 1992). However, the current data provide no evidence of any coronary dilator involvement. The possibility remains that the dilatation is as a result of the release of an endothelially derived mediator as previous evidence suggests that the endothelium is involved in the coronary dilatation in rat heart (Baydoun *et al.*, 1989). The possibility is still open that a hyperpolarising factor could be the agent released, although this was not confirmed in the current study. It would be of interest to repeat the glibenclamide perfusion experiments using a second set of controls in which the basal perfusion pressure had been raised (for example by manipulation of the flow rate of perfusate) in order to separate the effect of the increased basal perfusion pressure from the effect of glibenclamide on the ion channel.

The current data using the ET_A receptor-selective antagonist BQ-123, and the ET_B receptor-selective agonist sarafotoxin 6c, are consistent with the coronary constrictor response being mediated by ET_A receptors and the dilator response being mediated by ET_{B1} receptors. There was no indication from the present study of an ET_{B2} receptor-mediated vasoconstrictor effect (data from sarafotoxin 6c).

9.0 Calcium responses in smooth muscle cells in culture

The rise in intracellular calcium concentration ($[Ca^{2+}]_i$) is important for a number of functions including the contraction of smooth muscle and cell growth properties (see introduction, section 1.5). Extracellular and intracellular sources of calcium are both important in the regulation of $[Ca^{2+}]_i$, and both sources have been implicated in the responses to endothelins (van Renterghem *et al.*, 1988). Cells in culture are a frequently used and convenient model for the determination of calcium changes in response to extracellular stimuli, and this study examines responses to endothelins in smooth muscle cells derived and subcultured from fresh porcine coronary artery. The calcium responses were assessed by means of fluorescent calcium indicators, in preparations of cells in small groups of ~20 cells growing as a monolayer, in single adherent cells, and in populations of cells in suspension in a concentration of $1x10^6$ cells per ml.

Culture conditions (i.e. culture method, serum starvation and passage number) were kept to a similar protocol for experiments using all three types of measurement, as several phenotypic changes have been demonstrated according to culture conditions. For example, freshly isolated cells are differentiated and contractile (Chamley-Campbell *et al.*, 1979) and have a population of voltage operated calcium channels of mainly L-type (Neveu *et al.*, 1994). After exposure to serum, the cells become "modulated", cease to be contractile, and exhibit many features of proliferation. T-type calcium channels are the major type expressed in the modulated cell type (Neveu *et al.*, 1994). A return to the differentiated state can be achieved following an adequate period of serum starvation. Specifically for endothelin experiments, a change in the prominent receptor subtype has been demonstrated following the repeated passaging of cells (Eguchi *et al.*, 1994). Hence the standardisation of culture conditions was important for the comparison of the techniques used. In all types of experiment also, the agonist remained in contact throughout the measurement period in order to standardise the procedure, as this was unavoidable when using cells in suspension.

9.1 Monolayers of porcine coronary smooth muscle cells

The changes in $[Ca^{2+}]_i$ in a monolayer of cells were measured *in situ* without the need to remove the cells from the culture plate, and without loss of contact with neighbouring cells in a confluent monolayer. Basal $[Ca^{2+}]_i$ under these experimental conditions was 73±6nM (n=4), a level comparable to the results of others using monolayers of confluent smooth muscle cells in culture from a variety of different sources; examples of basal $[Ca^{2+}]_i$ levels, measured using fura-2 as an indicator, range from 73 to 115nM and smooth muscle cell sources include canine trachea (Yang *et al.*, 1994), rat aorta (Iijima *et al.*, 1991 ; Okada *et al.*, 1991) and human umbilical artery (Gardner *et al.*, 1992).

9.1.1 Calcium responses to endothelin-1

In cells grown in monolayers the addition of endothelin (10nM to 10μM) resulted in a rise in $[Ca^{2+}]_i$ followed by a plateau phase. This is consistent with the findings of several authors using monolayer preparations of smooth muscle cells (e.g. Iijima *et al.*,1991; Okada *et al.*, 1991; Gardner *et al.*, 1992; Okishio *et al.*, 1992; Yang *et al.*, 1994), in which agonist remained in contact with the cells.

In the current study, the initial increase in $[Ca^{2+}]_i$ in response to endothelin-1 was concentration-related in most preparations, but the size of the maximal responses was very variable (see figure R-23). The reasons for this are not clear, but the inter-preparation differences in maxima could reflect a different responsiveness in the hearts of different pigs from which the cells were cultured. If this is the case, then an increase in experimental number would be necessary in order to gain a clearer indication of the concentration relationship and the effect of inhibitors.

9.1.2 Effect of BQ-123 on responses to endothelin-1

In porcine and human coronary smooth muscle, the increase in vascular tone to endothelin has been attributed to both ET_A and ET_{B2} receptor activation (e.g. in man, Seo *et al.*, 1994; in pig, Fukuroda *et al.*, 1992, Schoeffter & Randriantsoa, 1993). Harrison *et al.*, (1992)

note vasoconstrictor effects in porcine coronary artery preparations which are attributable to neither subtype of receptor. Whether changes in $[Ca^{2+}]_i$ in porcine coronary artery smooth muscle cells are due to activation of ET_A or ET_{B2} receptors has not been approached. The competitive ET_A antagonist BQ-123 was used here in an attempt to clarify the receptor subtype through which the rise in $[Ca^{2+}]_i$ is mediated in this preparation. A 3 minute incubation of cells with BQ-123 at concentrations of 10nM and above successfully inhibited the $[Ca^{2+}]_i$ rise to endothelin-1 (see figure R-21 & R-22). When lower concentrations were used, however, no inhibition was apparent. This could be a reflection of the high degree of variability of the responses between preparations except that the individual cell preparations do not clearly show a concentration-related effect of BQ-123 at lower concentrations either. The effectiveness of the pentapeptide at higher concentrations is not simply a result of loss of cell viability, as the response to bradykinin was not altered at BQ-123 concentrations of 1-100nM. This also demonstrates the selectivity of the antagonism at the higher BQ-123 concentrations. The apparent "all or nothing" effect of the antagonist has not been previously reported, but BQ-123 has other properties which are not consistent with the action of a competitive antagonist.

The marked inhibition of the endothelin response at the 10nM BQ-123 concentration is clear and, within the limits of the concentration-response curve (Emax is not clearly attained), appeared to result in a reduction of maximal effect (figure R-22). The effects of BQ-123 and a closely related compound, BQ-153, as competitive antagonists have been well established in dose-contraction relationships in porcine coronary arterial strips, with a slope not significantly different from unity on Schild plotting (Ihara *et al.*, 1992). However, in experiments using monolayers of a neuroblastoma cell line, a non-competitive action of BQ-123 has been demonstrated (Hiley *et al.*, 1992). These authors suggest an uncoupling of the relevant G-protein from the receptor as a result of BQ-123 action, resulting in loss of $[Ca^{2+}]_i$ response. In rat brain capillary endothelial cells in culture, a similar non-competitive antagonism was seen with BQ-123, and this has been shown to be dependent on the time of interaction of the antagonist with the cells; simultaneous addition of BQ-123 and endothelin-1 resulted in a parallel shift of the concentration-response curve to the right

without affecting Emax, whereas preincubation of the cells with the antagonist resulted in a lower Emax (Vigne *et al.*, 1993). In the present study, monolayers were incubated with BQ-123 for a 3 minute period, which would allow time for a more stable antagonist-receptor complex to be formed as described by Vigne *et al.*, (1993). This phenomenon is reported only with BQ-123, the competitive effect of other antagonists being independent of the duration of incubation.

It is not possible to conclude that the inhibition seen with BQ-123 is a result of ET_A receptor antagonism or that the receptor is the only one involved in the increase in $[Ca^{2+}]_i$ in this preparation. If these experiments were repeated it would be advisable to employ a selective ET_A receptor antagonist which has been shown to act competitively in similar preparations such as FR139317 (McMurdo *et al.*, 1994).

9.1.3 Role of extracellular calcium in the peak and plateau phases of the endothelin response

The role of extracellular calcium entering via calcium channels was investigated using the divalent cation Ni^{2+} which is known to block calcium entry via voltage operated channels (Tsien *et al.*, 1988) as well as other, receptor-operated, calcium channels (Highsmith *et al.*, 1992). In the presence of 5mM $NiCl_2$ no significant change in the $[Ca^{2+}]_i$ increase to endothelin-1 was seen at either peak or plateau phase of the response, although there was an overall trend toward depression of both phases (figure R-25). It has been shown that extracellular calcium is needed for the prolonged response which is characteristic of endothelin-induced vasoconstriction (Hickey *et al.*, 1985; Yanagisawa *et al.*, 1988). Vasoconstriction to endothelins is attenuated in the presence of low extracellular calcium and in the presence of L-type calcium channel antagonists or Ni^{2+}, although this calcium entry is not confined to activation of L-type channels (van Renterghem *et al.*, 1988; Highsmith *et al.*, 1992). The effect of endothelin-1 on $[Ca^{2+}]_i$ in human vascular smooth muscle has been previously documented, and a partial dependence of both initial and late phases of the calcium response has been shown in a monolayer of cells (Gardner *et al.*, 1992). Hence an attenuation of both phases of the $[Ca^{2+}]_i$ increase would have been

expected in the presence of Ni^{2+}. The lack of significance shown here may, however, be a reflection of the variability between preparations, although it is likely that neither phase is totally dependent on the influx of extracellular calcium.

9.1.4 Sarafotoxin 6c effect on $[Ca^{2+}]_i$ in monolayers

The response to sarafotoxin 6c was subject to great variability, more so than the response to endothelins in monolayers of smooth muscle cells. In two preparations, no response was elicited from concentrations up to 10μM. In a further preparation a response at 5μM sarafotoxin 6c was gained, suggestive of a role for ET_{B2} receptors in the calcium rise in these cells, as this peptide is highly selective for ET_B receptors (Ki_{ETA} /Ki_{ETB} ~300,000; Williams *et al.,* 1991). In the responding preparation, an additional response could be elicited from endothelin-1 (1μm) after the response to sarafotoxin 6c had returned to basal level, but with sarafotoxin 6c remaining in contact with the cells. The reason for this has not been further examined here; the secondary increase may indicate that a separate receptor (probably ET_A) is involved in this response or that an increased number of the same receptor subtype are stimulated as a result of addition of a second agonist. If the initial response was a result of ET_{B2} stimulation, this would be consistent with previous findings (Fukuroda *et al.,* 1992; Schoeffter & Randriantsoa, 1993) in porcine coronary artery where both ET_A and ET_{B2} have been implicated in constriction. However, the number of preparations responding here to sarafotoxin 6c was low (1 of 3), which may indicate that the ET_{B2} receptor subtype is not present on all cells or in all hearts.

9.2 Calcium responses in single cells

The $[Ca^{2+}]_i$ increases in cells on coverslips were measured in single cells as part of a monolayer, by use of a narrow diaphragm to select the cell under microscopy. The $[Ca^{2+}]_i$ was calibrated using a similar technique to that for monolayers and this resulted in a similar reading for basal $[Ca^{2+}]_i$ levels, despite the warming of the cells to 37°C, which was not

done for the monolayers. Again, this level of calcium in unstimulated cells is consistent with results previously published (see section 9.1 above).

9.2.1 Responses to endothelin-1 in single smooth muscle cells

Endothelin-1 elicited a response in approximately 58% of cells (14 of 24). Where this response occurred, the peak increase in $[Ca^{2+}]_i$ was very variable, as may be seen in figure R-28. Some cells were unresponsive to endothelin-1 (10 of 24) although of these, 4 responded to another agonist, bradykinin. Thus, in a population of similar cells from a primary culture (with >99% identified as smooth muscle on staining), sub-populations may exist which are not able to produce a calcium response to a particular agonist. Whether this is due to a lack of the relevant receptor or to a failure of the coupling mechanism of that receptor to a calcium pathway is not clear. Vascular smooth muscle cells in culture have been shown to change in phenotype in many respects (Neveu *et al.*, 1994), the expression of receptors for endothelin being one of the factors affected (Eguchi *et al.*, 1994),. However, this particular change has been demonstrated at "late" passage (defined as passage 30-35), hence substantial change in subtype expressed is not expected in cells below 10 passages as used in the current study. It is possible, however, that individual cells in culture (in the "modulated" state - Chamley-Campbell *et al.*, 1979) may change in their receptor expression at different stages, and that these changes are not reversed after 24 hours of serum deprivation. These changes would only become apparent where single cells are examined. It is likely also, that a heterogeneous population of cells *in vivo* exhibit a variety of different characteristics which are not seen unless cells are examined individually. Different sensitivities of individual cells to a concentration of agonist have been demonstrated in bovine tracheal smooth muscle cells (not serum deprived - personal communication from K.Marsh) where, in the absence of extracellular calcium, the response to bradykinin was shown to be an "all or none" phenomenon (Marsh & Hill, 1993). However, the number of cells giving a uniform level of response was concentration dependent. These authors suggest a different sensitivity of individual cells to a particular agonist concentration. In the current situation, a further series of experiments using a

greater experimental number would be necessary to determine the number of cells responding to a single concentration of agonist. However, the responses to endothelin-1 do not conform to the "all or none" profile as the level of response was also variable.

9.2.1.1 Calcium oscillations in response to endothelin-1

There are few reports analysing the actions of endothelin in single cells, probably due to the high variability of latency, peak and subsequent phases of responses in these cells. Where individual cells have been used, however, there is no mention of oscillatory effects (e.g. Wagner Mann *et al.*, 1992; Wagner Mann & Sturek, 1992) although their measurement technique may not have allowed the time resolution required for their identification. The response of vascular smooth muscle cells to a single concentration of endothelin-1 was highly variable (see figure R-28) differing in both magnitude and in the oscillatory behaviour elicited. This is consistent with the findings of Linderman *et al.* (1990), in smooth muscle cells in response to ATP, where the oscillatory response is seen in 70-80% of cells and the type of oscillatory behaviour in individual cells varies greatly. The continued presence of the agonist was required for the oscillations to occur. In the study of Linderman *et al.*, (ibid), where the mean of the oscillatory calcium responses to ATP in 5 cells was calculated, the result was a peak and subsequent (noisy) plateau phase. The peak increase in $[Ca^{2+}]_i$ taken from larger cell numbers in a monolayer or in suspension, is thus likely to be dependent on the number of individual cells which respond, and will be the mean initial increase. The plateau phase is probably the summation of the oscillations in individual cells which are asynchronous.

9.2.1.2 Calcium oscillations in response to sarafotoxin 6c

The responses of individual cells to sarafotoxin 6c in different preparations were very variable, in a manner comparable with the endothelin variability. However, when a large increase in $[Ca^{2+}]_i$ in response to sarafotoxin 6c did occur, an oscillatory effect was produced. The selectivity of the toxin for ET_B receptors suggests that at least some of the cells responding were expressing the ET_{B2} receptor. This would be consistent with the

findings previously described using porcine coronary artery (e.g. Schoeffter & Randriantsoa, 1993; see section 9.1.4 above) where contractile responses were attributed to ET_{B2} activation. The response of a cell to endothelin-1 which did not respond to sarafotoxin 6c is suggestive that the ET_{B2} receptor is not present on all cells (see figure R-29).

9.3 Calcium responses in cell suspensions

The disadvantage of using cells in suspension is that some degree of disruption from normal function cannot be eliminated as loss of the extracellular matrix and potentially some disturbance of normal calcium equilibrium may have followed the dispersal procedure. The "normal" elongated cell conformation was also lost and the cells appeared rounded. However, use of such large numbers of cells should produce results which overcome the variability associated with heterogeneous sub-populations of cells; problems associated with different sensitivities and latencies between cells will be averaged thus producing a more distinct concentration-response relationship from which more information can be gained. A plateau response, if present, is likely to be a mean of the asynchronous oscillations produced in individual cells (see above, section 9.2.1.1).

The basal calcium levels produced in these populations of cells were significantly higher than the basal $[Ca^{2+}]_i$ in individual cells and monolayers. The basal $[Ca^{2+}]_i$ levels quoted in populations of cells in suspension are generally similar to those reported in monolayers and single cells (~100nM). However, in the current study, the basal measured $[Ca^{2+}]_i$ in cells in suspension was markedly higher than that in either monolayers or in single cells. Some allowance must be made for potential differences arising from the difference in calibration method (from the equation of Grynkiewicz *et al.*, 1985) rather than from a series of standard solutions. It is possible that some increase in apparent $[Ca^{2+}]_i$ may be due to an increase in membrane leakage following cell dispersal. It is likely that the dispersal

procedure would cause some degree of cellular activation and this would account for the elevated basal $[Ca^{2+}]_i$ levels. However, the cells used in suspension were all calibrated similarly and thus comparison may be made between controls and treated preparations from these experiments, if not in the absolute $[Ca^{2+}]_i$ between these and experiments using single cells and those used in monolayers.

9.3.1 Responses to endothelin-1 in cells in suspension

In suspension, porcine coronary smooth muscle cells gave a peak initial response to endothelin-1, which showed a concentration-related rise in $[Ca^{2+}]_i$. There was still a degree of variability in the peak responses between cells from different hearts, but this was not so marked as in the monolayers. A subsequent plateau component of the response was uniform at 200 seconds after the endothelin-1 administration, and unrelated to the concentration of endothelin-1 used. The lack of concentration-relationship in the plateau phase is interesting if this component of the response is seen as the combined effect of the highly variable oscillations in individual cells (Linderman *et al.*, 1990).

9.3.2 Effect of BQ-123 on calcium responses in cell suspensions

The lower (3nM) concentration of BQ-123 was ineffective in producing a significant inhibitory effect on the endothelin-1-induced calcium response. However, neither was the effect of the 10nM concentration as marked as that seen with the same concentration used in monolayers of cells following a 3 minute incubation. Simultaneous addition of the higher concentration of antagonist was performed to determine whether the apparent non-competitive effect of the pentapeptide seen in monolayer experiments was related to its duration of contact with the receptor as suggested by Vigne *et al.*, (1993). However, when used in suspensions of cells, there was no indication that the antagonist was acting in a non-competitive manner, which could indicate that the non-competitive appearance of the agent in the monolayer experiments was a spurious result and that the antagonism would have been overcome by use of higher concentrations of endothelin-1.

There has been a report (Sakamoto *et al.*, 1994) of a non-competitive BQ-123 antagonism in cells in suspension (in a mouse fibroblast cell line) after a 20 minute incubation with BQ-123, precluding the suggestion that the mode of culture was responsible for the type of antagonism seen. Further experiments would have been of use, using cells in monolayers with both simultaneous addition of antagonist and preincubation with BQ-123 to determine the effect of duration of contact.

9.4 Effect of herbimycin-A

Tyrosine kinase activity has been demonstrated in response to endothelin-1, in several cell preparations (Force *et al.*, 1991; Schvartz *et al.*, 1992) including vascular smooth muscle cells (Koide *et al.*, 1992a and b). The intention of these experiments using the tyrosine kinase inhibitor herbimycin-A, was to determine whether part of the endothelin-1-induced $[Ca^{2+}]_i$ rise could have been mediated via a tyrosine kinase-dependent pathway.

There is some evidence that tyrosine kinase activation (in response to a variety of agonists) may lead to an increase in $[Ca^{2+}]_i$ in smooth muscle and other preparations DiSalvo *et al.*, (1993) showed that contractile responses in taenia coli were reduced by inhibitors of tyrosine kinase. Inhibition of this enzyme using genistein has been associated with inhibition of voltage operated calcium currents in vascular smooth muscle (Wijetunge *et al.*, 1992) although direct action of the antagonist on the calcium channels was not ruled out. However, inhibition of calcium entry has also been shown in a rat basophilic leukaemia cell line using herbimycin-A (Teshima *et al.*, 1994); this inhibitor had a significant effect on the plateau phase of the $[Ca^{2+}]_i$ increase as well as on stimulated IP_3 increase in these cells, an effect separate from any action on the ion channel itself. Herbimicin-A inhibited tyrosine phosphorylation in the basophilic cell line as shown by Western blotting (*ibid*), at the concentrations eliciting the effects on IP_3 and $[Ca^{2+}]_i$.

A concentration of herbimicin-A (875nM) was used in the current study, which significantly inhibited both IP_3 and $[Ca^{2+}]_i$ increases in the experiments of Teshima *et al.*, (1994). This

agent was used in preference to genistein as the latter antagonist would interfere with the measurement of fura-2 fluorescence.

In herbimycin-A-treated suspensions of porcine coronary smooth muscle cells in culture, there was some reduction in the initial peak in $[Ca^{2+}]_i$ in response to endothelin-1, but this did not achieve significance (figure R-33). The plateau phase of the response to endothelin-1 was unaffected. It should be noted that the concentration of herbimicin-A employed here has not been shown to inhibit tyrosine phosphorylation in the smooth muscle cell preparations used. It is therefore possible that 875nM herbimicin-A was insufficient to cause inhibition of the enzyme in this smooth muscle cell preparation.

10 Summary of observations from calcium signalling experiments

Cell calcium measurements performed using fura-2 fluorimetry have been shown to be inconsistent between primary cultures of porcine coronary smooth muscle cells, when prepared in monolayers, suspensions or when investigated individually. This limits the number of absolute conclusions which can be drawn from the results here, but observations regarding the nature of the differences between the preparations used are summarised below.

Basal $[Ca^{2+}]_i$ appeared consistent in cells under microfluorimetry, at a level below 100nM, and corresponded to measurements described in the literature. However, in cell suspensions the basal $[Ca^{2+}]_i$ was significantly higher and the reasons for this could include leakage of the indicator into the extracellular space where the calcium concentration is 1mM. Little leakage of fura-2 would be necessary to be seen as a calcium increase above the nanomolar levels present within cells. Any cell damage could be the result of the dispersion procedure, which included stirring of cells to prevent aggregation (see methods section), and repeated centrifugation. Both of these procedures are a potential source of shear stress to the cell membranes.

Responses to endothelin-1 measured in the three types of cell preparation also displayed inconsistencies, both in the kinetics and in the amplitude of the responses. In all cases, the agonist remained in contact with the cells thus removing one variable cited by Wagner Mann & Sturek (1992). Within the monolayer experiments there was a large degree of variation in the amplitude of the responses between cells from different original coronary preparations. This could reflect a wide natural variability in the responsiveness to endothelins within a species. The suspensions of (larger numbers of) cells did not, however, show variability to the same extent, although this does not discount natural variation as an explanation for the differences between the monolayer preparations.

It is not possible to compare with confidence, the change in $[Ca^{2+}]_i$ in response to the agonist between suspensions and monolayers, as the basal $[Ca^{2+}]_i$ difference may indicate

also a difference in the calibration. However, the form of the responses may be compared between all three preparations.

Peak and subsequent plateau phases of the increase in $[Ca^{2+}]_i$ in response to endothelin-1, evident in cells in suspension, were also present in most responses in monolayers. However, in single cells no examples of a plateau phase were seen and instead, an oscillatory form of response was produced. The oscillations lacked consistency between cells, in amplitude and frequency.

Another problem regarding the consistency of measurements in single cells, was the number of individual cells unresponsive to an agonist. The agonist to which a cell would respond and the amplitude of response were not predictable. This could suggest that subpopulations of cells were present which possessed different capabilities of response, either through different expression of receptors or through variations in the signal transduction capacities following receptor stimulation. These could include expression of different ion channel populations or intracellular enzymes in "modulated" as opposed to differentiated cells (Chamley-Campbell *et al.*, 1979; Neveu *et al.*, 1994). Use of a considerably higher experimental number of cells would be necessary before conclusions about the concentration-response relationship could be drawn.

Investigation into the subtype of receptor involved in the calcium increase in these smooth muscle cells produced no conclusive evidence of either ET_A or ET_{B2}. The inhibitory effect of the ET_A receptor antagonist BQ-123, was of different appearance in the suspensions when compared with cell monolayers. Inhibition of the endothelin-1-induced rise in $[Ca^{2+}]_i$ was significant in monolayers at a 10nM concentration, whereas at a similar concentration in cell suspensions, there was no significant effect. The results in monolayers were somewhat unusual, also. The appearance of the inhibition was that of a non-competitive antagonist, and a similar effect has been reported previously although in different cell types (Hiley *et al.*, 1992; Vigne *et al.*, 1993). The apparent "all or none" effect of the inhibitor has not been reported previously and would require further investigation. Higher concentrations of BQ-123 would be required to investigate whether a similar unusual effect occurred in cell suspensions.

Other experiments using the ET_B receptor agonist sarafotoxin 6c were inconclusive, with some preparations responsive and others unresponsive. This was also apparent in the responses of single cells. The subtype of receptor expressed could be a result of cell culture conditions which were similar for each type of preparation, regarding the passage number and duration of serum deprivation. These conditions could have favoured a predominance of one receptor subtype over another and may not reflect the ratio of receptors existing *in vivo* or in preparations where contraction has been shown to be mediated by both receptor subtypes in porcine coronary artery (e.g. Schoeffter & Randriantsoa, 1993).

Finally, attempts to discover any role for a tyrosine kinase dependent increase in $[Ca^{2+}]_i$ were inconclusive, no significant difference in controls and herbimycin-A-treated preparations being found.

11 Future work

11.1 Ischaemia reperfusion experiments

A further line of enquiry arising from the original publications of the group reporting the increased binding of endothelin-1 to cardiac membranes, would be the investigation of the effect of amlodipine pretreatment. Comparison of the current results with the effects of ischaemia / reperfusion in hearts from amlodipine-treated rats would be of interest as a similar protocol was shown to prevent the increase in binding site density which otherwise followed ischaemia / reperfusion (Nayler *et al.*, 1992).

Another point which could be addressed arising from the same source, is the origin of the cardiac membranes on which binding density was increased. This increased binding could equally well be on cardiomyocytes as on coronary smooth muscle. A different model in which it is possible to compare the effects of the ischaemia / reperfusion protocol used by Liu *et al.*, (1991) would be required. One such model would be isolated papillary muscle and atrial preparations from ischaemic / reperfused hearts, where increased myocardial reactivity might be apparent. A separate model would be an isolated myocyte preparation in which fluorimetric measurement of calcium increases could be compared from control and from ischaemic / reperfused hearts It would be technically difficult to ensure that the ischaemia / reperfusion conditions resembled those in the original paper, so parallel binding studies would be needed to confirm that the increase in binding site density was a feature of the protocol used.

The investigation of the mechanism underlying the vasodilator component of the endothelin and sarafotoxin 6c responses in the isolated perfused rat heart was not completed, as the purpose of the experiments was to diminish vasodilatation with reliability. However, the full mechanisms could be examined initially by use of separate inhibitors of cyclooxygenase and nitric oxide synthase in the perfusate in a range of concentrations, including higher ones than those employed to date.

11.2 Cell signalling experiments

The reasons for the differences in responsiveness between individual cells from the same preparation are of concern. If the cell culture conditions are responsible for the production of subpopulations of cells of variable degrees of differentiation, then it would be of value to optimise the conditions producing a more homogeneous cell population. The study by Neveu *et al.*, (1994) showed that, although serum deprivation significantly reduced the rate of appearance of new cells after 24 hours, a longer period could be necessary to maximise the numbers of cells displaying a differentiated phenotype. This could affect many parameters including receptor and ion channel expression. Comparison between cells deprived of serum for longer periods such as 48 and 72 hours would be of value. Measurement of [^{3}H]-thymidine incorporation rate could be used to determine the rate of cell division and thus indicate the degree of quiescence of a population. Many experiments are performed using transformed smooth muscle cell lines which do not become differentiated, and these could be compared with quiescent cells using a similar protocol. It would also be necessary to compare non-cultured, freshly prepared cells with those in culture. These would be more physiologically representative although the enzymatic dispersion process has its own drawbacks.

Ideally, a population of cultured cells in a monolayer could be compared with cells *in situ*, by use of the front surface fluorimetry technique (see introduction). These cells *in situ* are little disturbed from their physiological state and may more closely reflect the effect of an agonist in vivo.

Once cell culture conditions were optimised, further experiments could be performed with greater confidence. The different manifestation of the inhibitory effect of the antagonist BQ-123 on monolayers when compared with cells in suspension is worthy of further study. The comparison of cell calcium responses in monolayers preincubated with BQ-123, with responses where the antagonist was added simultaneously with endothelin-1 would indicate whether competition was related to duration of contact as suggested by Vigne *et al.* (1993).

The difference in the manifestation of inhibition could be further studied using a greater range of BQ-123 concentrations in both preparations with the aim of showing competition by Schild plots. For analysis of the receptor subtype involved, a better method of study would be the use of selective ET_A and ET_B receptor antagonists which are not known to display different kinetics after longer contact with the cells. These could include the ET_A selective agonist FR 139317 and the novel ET_B antagonist BQ-788.

In the investigation of a role for tyrosine kinase in the rise in $[Ca^{2+}]_i$, a series of experiments using a range of herbimycin-A concentrations would be of greater value. These could be performed in parallel with Western blotting experiments using anti-phosphotyrosine antibodies (such as PY20) to confirm the effect of the concentrations used on tyrosine phosphorylation.

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